NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1995

Submitted by

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Project Director

Period Covered: 20 September, 1994, through 19 September, 1995

Cooperative Agreement DAMD17-93-V-3013

between

United States Army Medical Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center
Neuroscience Center of Excellence

Volume 8 of 8

Protecting the Auditory System and Prevention of Hearing Problems

Project Directors:
Richard Bobbin, PH.D.
Charles Berlin, Ph.D.

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ANIMAL USE
20 SEPTEMBER, 1994, THROUGH JULY, 1995

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Protecting the Auditory System and Prevention of Hearing Problems**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC #
guinea PIG	99	91	1061

Investigator Signature

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5. ABSTRACT: (SPECIFIC AIMS) (Maximum of 200 words)

ANIMAL PROJECT: The **SPECIFIC AIMS** of this study are to demonstrate and explore mechanisms for preventing the effects of intense sound. In years 01 and 02 we discovered that continuous, ipsilateral primary stimulation (CM-LIPS) will produce complex changes in the cochlea and that L-type Ca^{2+} channels are involved. We discovered that exposure to a very low level continuous noise (64 dB SPL for 10-11 days) results in changes in cochlear partition mechanics and in the response of OHCs (and possibly the supporting cells) to ATP.

HUMAN PROJECT: Major findings since last year include: (1) binaural stimulation with tone bursts generates more than three times the emission suppression of ipsilateral or contralateral stimulation in a forward masking paradigm when the stimuli are below 70 dB SPL; (2) Females show larger emissions than males; (3) Using tone bursts we can show more controllable and measurable emission than using broad band clicks. Twenty musician subjects are in different stages of study at present, and 20 more are being sought.

6. INTRODUCTION (Hypothesis): The following text is approximately the same as in the Progress Report for year # 01. The nature of the problem that this proposal addresses is that soldiers may be exposed to intense noise hazards which will affect their hearing. The hypothesis to be tested is that noise induced hearing loss can be prevented or attenuated.

The literature suggests that the efferent nerve fibers synapsing on the outer hair cells (OHCs) in the cochlea may attenuate the effects of intense sound. One example of this may be the demonstration that chronic low level sound will "toughen" ears so subsequent intense noise will induce less damage (e.g., Canlon et al., 1988; Campo et al., 1991; Franklin et al., 1991). We demonstrated that sound will activate cholinergic efferent fibers leading to the cochlea (Kujawa et al., 1994a). The released acetylcholine (ACh) hyperpolarizes the OHCs (Erostegui et al., 1994) which in turn attenuates the mechanical response of the cochlear partition to sound as measured by distortion product otoacoustic emissions (DPOAE; Kujawa et al., 1992, 1993, 1994a). These results predict that low level sound will activate the efferents and consequently will reduce the effects of intense sound by attenuating cochlear partition motion. Whether this occurs in "toughening" remains to be determined. In years #01 and #02 we have obtained data indicating that not only ACh but ATP may also play a major role in noise effects in the cochlea. ATP is a putative neuromodulator in the cochlea. It may be released together with ACh from the efferents or it may be released from

other cells in the cochlea. ATP may be a major player in the effects of toughening because it has powerful effects on the OHCs and supporting cells and ATP is known to induce cell death (Bobbin, 1995; Eybalin, 1993; Kujawa et al., 1994b; Valera et al., 1994). Whether the release of ATP induces cell death in the cochlea remains to be explored.

The human experimentation program was proposed to develop emission-based tests to detect abnormal cochlear function rapidly and accurately. The procedure offers the equivalent of a non-invasive acoustic microscope to analyze the integrity of the OHCs; this is an important tool since OHC damage is always seen in humans who have suffered noise damage. The hypothesis is that "noise tender" ears that are particularly susceptible to noise damage will show different emission suppression patterns from ears that are "tough".

Our intention remains to study 100 musicians and industrial workers who have had matched exposures to noise in an efferent suppression paradigm. Before starting the final design of this study we laid basic parametric groundwork relative to the nature of the stimuli, their delivery (whether clicks or tones, whether binaural, contralateral only, or ipsilateral), the echo analysis, and whether there are any pertinent gender and ear differences which have to be entered into the final subject selection.

In summary, in animals we will test the hypothesis that: (1) the impact of noise on hearing can be lessened; (2)

certain classes of drugs may prevent (or exacerbate) noise-induced hearing loss. In human subjects, we will explore the concept that some individuals are more or less susceptible to noise damaging effects. We will describe those populations to determine the basis of this "toughness" or susceptibility to damage from noise. We will examine whether the techniques which we may discover aid in preventing noise-induced hearing loss in soldiers.

7. BODY (STUDIES AND RESULTS CONDUCTED UP TO AND DURING THE CURRENT BUDGET YEAR 02):

ANIMAL PROJECT: In years #01 and #02, we have continued to carry out acute and chronic experiments as described in the year #01 progress report. In brief, the acute experiments focus on the effects of ipsilateral sound and the chronic experiment examined "toughening" together with whole cell voltage clamp experiments. These experiments follow directly our stated specific aims for years #1 and #2 and the first three TECHNICAL OBJECTIVES: to extend studies which demonstrate that contralateral, ipsilateral, or "toughening" sound will prevent the effects of intense noise, to test the role of the efferents, and to explore cellular mechanisms.

The methods were described (Kujawa et al., 1992; 1993; 1994a; year #01 progress report). Briefly, guinea pigs are anesthetized (urethane: 1.5 gm/kg) and tracheotomized. ECG is monitored and temperature maintained at 38° ± 1°C. The right auditory bulla is exposed, opened and tendons of the middle ear

muscles are sectioned. For drug application to the cochlea, holes are placed in the cochlear basal turn: one in scala tympani for the introduction of perfusates and one in scala vestibuli to allow fluid escape. Perfusates are introduced into scala tympani at approximately 2.5 μl/min for 10 min through a pipette coupled to a syringe pump. DPOAEs ($2f_1-f_2$ & f_2-f_1) are recorded in response to primary stimuli ($f_2/f_1=1.2$) delivered to the right ear of each animal by an acoustic probe/hollow ear bar assembly. Acoustic signals present within the canal are detected by a microphone system (Etymotic Research, ER-10) contained within the probe. Microphone output is directed via a preamplifier (Etymotic Research, ER-1072) to a signal analyzer (Hewlett Packard 3561A). To extract the DPOAE from the canal spectrum, the signal is sampled, digitized and submitted to Fast Fourier Transform (FFT) analysis. The resulting spectrum is averaged (over 25 samples) and displayed at the spectrum analyzer (1 kHz window, 3.75 Hz BW). DPOAE amplitude is defined as the spectral peak corresponding to the DPOAE frequency. The contralateral stimulus is a wideband noise with an overall level of 70 dB SPL and flat from 0.9 to 15.8 kHz.

The acute experiment examined the influence of continuous, moderate-level (60 dB SPL) ipsilateral primary stimulation (CM-LIPS) on the f_2-f_1 DPOAE at 1.25 kHz. The stimulation and response monitoring protocols consisted of quiet for 15 min, then turning the primaries on for 9 min, off for 1 min and then on for an additional 3 min. CM-LIPS resulted in a complex

change in the magnitude of f_2-f_1 (see Appendix 1 for publication). In year #02 we continued to explore mechanisms for the CM-LIPS-induced alteration in mechanics. We first ruled out whether efferent nerves were inducing these changes. The results have recently been published (Appendix 1).

In addition during year #02, we examined the role of calcium and L-type calcium channels by examining the effects of BAPTA, calcium channel antagonists (magnesium and nimodipine) and an agonist, BayK. All reversed the polarity of the "off-effect" and had complex effects on the overall shape of the response indicating that calcium plays an important role in part of the CM-LIPS-induced alteration in mechanics. These results are in a manuscript being prepared for publication (Appendix 2).

As described in year #01 (See PROGRESS REPORT for year #01), in the chronic experiment, the "toughening" sound used was a continuous low-level band of noise (85 dB SPL, band centered at about 1.5 kHz with 3 dB cut off at 1.025 and 2.125 kHz) which is similar to that used by others (e.g., Henderson, 1994). When the study was complete, several colleagues suggested to us that the noise was measured in a non-standard method. We used peak SPL on the open scale (85 dB SPL) and the more accepted method is rms SPL on A scale (64 dB SPL). Because the level of the noise was very low (64 dB SPL), fellow scientists were skeptical. They stated that the results we obtained were due to sampling error when we presented the preliminary results indicating that the toughening sound induces a TTS by suppressing DPOAEs the annual meeting of

auditory scientists (Crist et al., 1995, Appendix 3; Skellett et al., 1995a, Appendix 4). At the time we had only carried out an N of 5 animals per data point and so sampling error was a real possibility. Therefore, during year #02 we replicated the study utilizing an N of 13 animals per data point and reducing the monitoring days to days 0, 3 and 11. The results duplicated the previous results on the $2f_1$-f_2 DPOAE. Thus, we are the first to demonstrate that low level sound exposure induces changes in the mechanics of the cochlea and this may underlie toughening. A manuscript is being prepared for publication (Skellett et al. 1995b; Appendix 5). However, we are now in the process of exposing animals to a more intense sound (our original intended 85 dB SPL rms, A-scale) to test whether toughening occurs with the intense sound and are comparing continuous to intermittent exposures (experiment #3 in the original proposal). In addition, we are testing whether the low intensity (64 dB SPL) sound induces toughening as monitored by a decrease in the effects of a subsequent intense sound exposure.

As reported in year #01 Progress Report, to examine the cellular basis for these "toughening"-induced changes in the cochlea (experiment #4 in original application), we carried out whole cell voltage clamp recordings from the OHCs of these animals after the DPOAE recordings were made. The results indicated ATP receptors have down-regulated (i.e., become less in number) during "toughening". This may be the molecular mechanism for "toughening". A manuscript is in press (Chen et al., 1995;

Appendix 6). During year #02 we have demonstrated that rat OHCs do not respond to ATP but that both rat and guinea pig supporting cells do respond to ATP and we have described the current characteristics of the response and the pharmacological classification of the receptor type. These results are a totally new discovery and during year #03 we will explore the role of the supporting cells (Deiter's cells) in noise induced hearing loss and in toughening. In addition, we are attempting to detect which cells in the cochlea release the ATP during sound exposure.

HUMAN PROJECT:

In our previous progress report we noted that we needed new hardware to overcome the idiosyncrasies of the available ILO88 systems. The manufacturer and designers of the equipment were most helpful and collegial in helping us work around some of the limitations imposed by their design. However, when we found we needed single click stimuli with control of masker-stimulus intervals in 1 msec increments, they informed us that their system could not meet our requirements. Therefore we built our own.

Hardware For this period we have completed the construction of the first of two unique proprietary otoacoustic emission evaluation and testing systems. Han Wen, our biomedical engineer, has designed and built a Lab-View IBM-based otoacoustic emissions system we have named Echo-Lab. Figs. 1-4 (See appendix 8) show the various displays and protocols that are possible with this system. A new system was required to allow us to use our unique measuring system with a file structure compatible with the ILO88

systems on which all of our previous data were collected. The new system has these features:

1) Allows one click at time to be studied: First, the system allows us to use click and tone-burst evoked emissions one at-a-time instead of in groups of 4 which was the only available format in the ILO88. It was important for us to be able to use clicks and tone bursts one-at-a-time in order to have precise timing control over our stimuli to see if the duration over which suppression stretched would be in some way related to "toughening of the ears".

2) Allows intervals between masked and noise to be varied in one msec steps: The ILO88 allowed only variation in multiples of 20 msecs which proved too gross a step for our purposes.

3) Allows tone bursts and narrow bands of noise to be studied binaurally, ipsilaterally as well as contralaterally: The ILO88 also allowed binaural, ipsilateral and contralateral signal presentation, but had limited control over the bandwidth of any of the stimuli, thus precluding the study of signal bandwidth as a variable in ear toughening experiments.

4) Allows custom configuration of the click to assure a flat spectrum in every ear used for the experiments: The ILO88 reflects the ear canal spectrum but requires some compromise with each subject so that not every subject will receive the same absolutely flat click spectrum. Our system allows a cepstral re-convolution of the click relative to the initial spectrum so that the experimental stimulus is always flat in the ear. (See Fig. 4,

Appendix 8).

New Developments, Subjects and Experiments Since the last Report

Staff: Jill Bordelon who is an outstanding audiologist, with the highest performance ratings, has been hired to replace Dena Fair Jackson.

Musicians Dr. Berlin, who is a member of the Musician's Local here in New Orleans, arranged to meet with the Board of Directors of the Union and got them to agree to put an announcement in the monthly Newsletter re: our need for subjects for these experiments. As a result of this notice 8 musicians applied initially, and were tested audiologically and with emissions. Seven subjects met the criteria and have been accepted into the project. Thirteen music students and faculty from Loyola University have also been tested and tentatively enrolled through the courtesy of our colleagues Drs. L. Christenson and S. Hinderlie. In addition, a number of world-famous performers (eg. Neville Brothers, H. Connick, Jr.) have expressed interest in participating but have unpredictable travel schedules and will have to be "worked in" on an ad hoc basis. Although we are beginning on our first 20 subjects, a second and third follow-up notice will go out shortly to meet an additional target of 20 more subjects. We plan to expand to the military subjects after our second system is built and we can solidify our data collection protocols and teach them to others.

Experiments on Normals: Two new procedures have been instituted:

a. Each subject will have his/her ears cleaned and a custom silicone earmold made. That mold will be used for all experiments to insure comfort and identical fit from condition-to-condition.

b. All data will be collected on the new Echo-Lab system and, if comparable data are needed from an ILO 88 system, they will be collected on *both* systems and compared.

We have begun a new series of Experiments using Echo-Lab's Tone-bursts and narrow bands of noise. Pilot data comparing 1/2 octave to 1, 1.5 and 2 octave band wide noises revealed that the best results would be seen with 1500 Hz Blackman envelope 4 msec rise-time tone bursts and one-octave-wide noise centered at 1500 Hz.

Data in Fig. 5 (Appendix 8) show results on 7 normal subjects which confirm both the relative power of the binaural condition and the comparability of the one-octave band results to the two-octave band findings. Data in Fig. 6 (Appendix 8) show threshold and amplitude data for emissions elicited by 1500 Hz tonebursts which were used to help us decide on the values to be used in the next experiments.

Our experiments on the musicians will therefore use these conditions with tone bursts set at 55 dB SPL and one-octave band wide noise set at 65 dB SPL.

8. CONCLUSIONS (PLANS FOR YEAR 3 OF SUPPORT):

ANIMAL PROJECTS: The completed research indicates that guinea pig cochlea mechanics and the response of OHCs to ATP are altered significantly by the use of low level continuous sound, a level of sound previously believed not to induce changes in the ear. Presently and in future work during year #03, we will examine whether this low level (64 dB SPL) of continuous sound and higher levels (84 dB SPL) of sound induce "toughening" as monitored by a reduced response to a subsequent intense sound exposure. The cellular mechanisms for the "toughening" appears to involve ATP, OHCs and supporting cells. In future experiments (year # 03) we will further explore the effect of noise exposure and toughening on the OHC and supporting cell responses to ATP. In addition, ATP will be administered into the cochlea to examine whether ATP mimics the cell death observed in noise exposure. ATP antagonists will be used to attempt to block noise damage, addressing objectives #3, #4 and # 5 in the original proposal. The molecular mechanisms for ATP action is being explored by monitoring the effects of ATP on cell currents and on intracellular calcium levels utilizing the new confocal microscope in the Core laboratory. ATP-induced changes in cell calcium may be the final pathway whereby "toughening" or noise exposure affects the cochlea.

HUMAN PROJECT: In view of Dr. Bobbin's findings on low level "toughening" we will also query our subjects about the non-occupational noise and sound exposure they undergo. We will also introduce attempts at recording the f2-f1 DPOAE in some of our

subjects and compare these data, and their degree of contralateral suppression, to the more commonly recorded 2f1-f2 DPOAE.

In our previous report we wrote:

"We are also developing our own proprietary system which can produce and analyze exactly the type and time-sequence of stimuli we would like without the intrusive three-memory dead space..."

This system is now operational and has been bench tested extensively. It will be used in the following studies:

Tone burst studies without the "dead space" discussed in our previous application, have been instituted with our new Echo Lab system. We will use a 1500 Hz Hamming tone burst and one Octave band wide 400 msec noise, 5 dB more intense than the tone burst for our binaural, ipsilateral and contralateral forward masking studies. We also plan a continuous contralateral noise study.

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10. List of publications and abstracts:

a). Kujawa, S.G., Fallon, M., and Bobbin, R.P. Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. I. Response characterization and contribution of the olivocochlear efferents. **Hear. Res.** 85: 142-154, 1995.

b). Bobbin, R.P. Kujawa, S.G., Skellett, R.A., and Fallon, M. (1995) Nimodipine, BayK, and lowering Ca^{2+} levels in perilymph alter distortion product otoacoustic emissions. In Preparation.

c). Crist, J.R., Skellett, R.A., Fallon, M. and Bobbin, R.P. Changes in distortion product otoacoustic emissions following continuous noise exposure. **Abstracts of the 18th Midwinter Research Meeting, Association for Research in Otolaryngology,** St. Petersburg Beach, Florida, abstract 297, February 5-9, 1995.

d). Skellett, R.A., Crist, J.R., Fallon, M. and Bobbin, R.P. The effect of continuous moderate-level noise exposure on contralateral suppression. **Abstracts of the 18th Midwinter Research Meeting, Association for Research in Otolaryngology,** St. Petersburg Beach, Florida, abstract 298, February 5-9, 1995.

e). Skellett, R.A., Crist, J.R., Kujawa, S.K., Fallon, M. and Bobbin, R.P. (1995b) Chronic low-level noise exposure alters distortion product otoacoustic emissions. In Preparation.

f). Chen, C., Nenov, A., and Bobbin, R.P. Noise exposure induced

change in outer hair cell response to ATP. **Hear. Res.** In Press, 1995.

g). Kujawa, S.G., Fallon, M., and Bobbin, R.P. A suppressive "off-effect" in the f_2-f_1 DPOAE response to continuous moderate-level primary stimulation. **J. Acoust. Soc. Am.,** 95, 2845, 1994.

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i). Berlin, CI., Hood, LJ., Hurley, AE., Wen, H., and Kemp, DT. Binaural Noise suppresses linear clicks more than ipsilateral or contralateral noise. **Hear. Res,** In press, 1995.

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13. Inventions and Patents

None

14. List of Appendicies

Appendix 1. Kujawa, S.G., Fallon, M., and Bobbin, R.P. Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. I. Response characterization and contribution of the olivocochlear efferents. **Hear. Res.** 85: 142-154, 1995.

Appendix 2. Bobbin, R.P. Kujawa, S.G., Skellett, R.A., and Fallon, M. (1995) Nimodipine, BayK, and lowering Ca^{2+} levels in perilymph alter distortion product otoacoustic emissions. In Preparation.

Appendix 3. Crist, J.R., Skellett, R.A., Fallon, M. and Bobbin, R.P. Changes in distortion product otoacoustic emissions following continuous noise exposure. **Abstracts of the 18th Midwinter Research Meeting, Association for Research in Otolaryngology**, St. Petersburg Beach, Florida, abstract 297, February 5-9, 1995.

Appendix 4. Skellett, R.A., Crist, J.R., Fallon, M. and Bobbin, R.P. The effect of continuous moderate-level noise exposure on contralateral suppression. **Abstracts of the 18th Midwinter Research Meeting, Association for Research in**

Otolaryngology, St. Petersburg Beach, Florida, abstract 298, February 5-9, 1995.

Appendix 5. Skellett, R.A., Crist, J.R., Kujawa, S.K., Fallon, M. and Bobbin, R.P. (1995b) Chronic low-level noise exposure alters distortion product otoacoustic emissions. In Preparation.

Appendix 6. Chen, C., Nenov, A., and Bobbin, R.P. Noise exposure induced change in outer hair cell response to ATP. **Hear. Res.** In Press, 1995.

Appendix 7. Kujawa, S.G., Fallon, M., and Bobbin, R.P. A suppressive "off-effect" in the f_2-f_1 DPOAE response to continuous moderate-level primary stimulation. **J. Acoust. Soc. Am.**, 95, 2845, 1994.

Appendix 8. Figures for the human studies.

Appendix 9. Berlin, CI., Hood, LJ., Hurley, AE., Wen, H., and Kemp, DT. Binaural Noise suppresses linear clicks more than ipsilateral or contralateral noise. **Hear. Res**, In press, 1995.

APPENDIX #1
DAMD17-93-V-3013
Bobbin and Berlin

ELSEVIER

Hearing Research 85 (1995) 142–154

HEARING RESEARCH

Time-varying alterations in the f_2–f_1 DPOAE response to continuous primary stimulation I: Response characterization and contribution of the olivocochlear efferents ☆

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Received 18 October 1994; revised 2 February 1995; accepted 18 February 1995

Abstract

The f_2–f_1 distortion product otoacoustic emission (DPOAE) can be observed to undergo gradual alterations in amplitude during continuous ipsilateral stimulation with primary tones. In the present experiments, we characterized the dependence of these amplitude alterations on several stimulus variables (intensity, duration, frequency) and on DPOAE type (quadratic vs cubic) and tested the hypothesis that such alterations are mediated by the olivocochlear (OC) efferents. Responses were recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles before and after intracochlear application of antagonists (curare, 1 μM; bicuculline, 10 μM; tetrodotoxin, 1 μM) or before and after OC efferent section at the midline of the floor of the IVth ventricle. We confirm previous reports of continuous stimulation-related alterations in the amplitude of the quadratic distortion product, f_2–f_1, and report a novel, suppressive 'off-effect' apparent in f_2–f_1 amplitude following a short rest from such stimulation. Response alterations were sensitive to primary intensity and to duration of rest from continuous stimulation, but were not clearly frequency-dependent over the ranges tested. Corresponding alterations in the amplitude of the cubic nonlinearity, $2f_1$–f_2 were very small or absent. Amplitude alterations in f_2–f_1 were reduced but not blocked by OC efferent antagonists (curare, bicuculline) and were largely unaffected by TTX or by midline brainstem section. All of these manipulations, however, prevented completely the known efferent-mediated contralateral sound suppression of both f_2–f_1 and $2f_1$–f_2 DPOAEs. Taken together, these results do not provide support for efferent control of the f_2–f_1 amplitude alterations observed during continuous ipsilateral stimulation.

Keywords: Olivocochlear efferents; Otoacoustic emissions; Outer hair cells; Quadratic nonlinearity; Cubic nonlinearity

1. Introduction

The quadratic nonlinearity, f_2–f_1, measured as a distortion product otoacoustic emission (DPOAE), displays time-varying amplitude alterations during continuous, primary tone stimulation. Brown (1988) first described a short period of amplitude growth followed by large (often exceeding 15 dB) reductions in f_2–f_1 DPOAE amplitude during continuous low- to moderate-level primary stimulation. Similar amplitude alterations were not observed in the cubic nonlinearity corresponding to the frequency $2f_1$–f_2. In a subsequent investigation, Whitehead et al. (1991) also observed f_2–f_1 but not $2f_1$–f_2 to undergo an initial amplitude increase followed by a gradual decline during continuous primary stimulation. Rarely exceeding 3 dB, the magnitude of the f_2–f_1 amplitude reduction was much smaller than that observed by Brown. Kirk and Johnstone (1993) confirmed the perstimulatory reductions in f_2–f_1 amplitude and reported effects to be most robust within the primary frequency range 2–7 kHz. Again, corresponding changes in the $2f_1$–f_2 distortion product were minimal or absent at any of the frequency combinations tested.

☆ A preliminary report was presented at the 127th Meeting of the Acoustical Society of America in June, 1994.

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SSDI 0378-5955(95)00041-0

In contrast to the different behavior of these distortion components during ipsilateral stimulation, both f_2-f_1 and $2f_1-f_2$ distortion products can be altered during presentation of tones or noise to the contralateral ear (e.g., Kirk and Johnstone, 1993; Kujawa et al., 1993, Kujawa et al., 1994; Puel and Rebillard, 1990). When appropriate measures are taken to avoid transcranial crossover of the contralateral signal or middle ear muscle activation, these contralateral influences on ipsilateral DPOAEs reveal clearly an efferent influence on the cochlear mechanics. This efferent control is thought to be mediated primarily by uncrossed medial olivocochlear (UMOC) neurons which synapse directly with outer hair cells (OHCs) and which, due to the predominantly crossed nature of the afferent pathways, are activated primarily by contralateral sound (Warren and Liberman, 1989). Such response suppression can be mimicked by electrical activation of OC neurons (Kirk and Johnstone, 1993; Mountain, 1980; Siegel and Kim, 1982). It can be prevented by cuts to the brainstem that remove this efferent input (Puel and Rebillard, 1990; Puria et al., 1992) and it can be blocked pharmacologically by antagonists of OC efferent activity (Kujawa et al., 1993; Kujawa et al., 1994).

The mechanisms underlying the f_2-f_1 amplitude alterations during continuous ipsilateral stimulation are unknown. Brown (1988) and Kirk and Johnstone (1993) have presented results suggesting that the ipsilateral effects, too, are under efferent control. In the Brown (1988) experiments, f_2-f_1 response alterations were absent or reduced in magnitude in deeply-anesthetized animals. Additionally, they were altered by the ipsilateral presentation of 'novel' stimuli and by stimulation of the contralateral ear during periods of rest from ipsilateral stimulation. In the Kirk and Johnstone (1993) studies, the amplitude alterations were prevented in some animals by intracochlear application of bicuculline, an antagonist of GABA, a putative transmitter of apical cochlear efferents (see Eybalin, 1993 for review) and by tetrodotoxin (TTX). The amplitude alterations were not, however, blocked by strychnine, which has potently antagonized every known OC efferent-mediated effect identified to date (e.g., Bobbin and Konishi, 1974; Desmedt and Monaco, 1962; Kujawa et al., 1994). Yet, in these same animals, strychnine blocked contralateral suppression of f_2-f_1 and abolished the suppressive effects of electrical OC stimulation on both the compound action potential (CAP) of the auditory nerve and the f_2-f_1 DPOAE. Moreover, Whitehead et al. (1991) found no support for efferent control of these amplitude alterations in rabbit. Specifically, responses were not different in awake and anesthetized animals, and they were not altered by contralateral sound. Finally, none of these investigations have revealed corresponding alterations in the amplitude of the $2f_1-f_2$ DPOAE. Given the similar behavior of the f_2-f_1 and $2f_1-f_2$ DPOAEs in response to efferent activation by contralateral sound, it remains unclear why ipsilateral sound-activation of efferent neurons should affect f_2-f_1 and not $2f_1-f_2$ DPOAEs.

Our ultimate goal in these experiments is to identify the mechanism(s) underlying the f_2-f_1 DPOAE amplitude alterations observed during continuous primary stimulation. In this paper, we confirm the amplitude alterations observed by others and report, in addition, a suppressive 'off-effect' of continuous stimulation not described in previous reports. We describe the dependence of these amplitude alterations on several stimulus variables (level, duration and frequency) and on DPOAE type (quadratic vs cubic). In addition, we present results related to our tests of the hypothesis that the amplitude alterations are under efferent control. Toward this end, we studied two antagonists of OC efferent activity (curare, bicuculline) that we have found previously to block contralateral sound suppression of the $2f_1-f_2$ DPOAE (Kujawa et al., 1994), an antagonist of all action potential-mediated activity (TTX) and OC nerve section for their effects on these time-varying changes in f_2-f_1 amplitude. Contralateral suppression of f_2-f_1 and $2f_1-f_2$ DPOAEs was employed as an internal, known efferent-mediated control for the effects of these experimental manipulations.

2. Methods

2.1. Subjects

Pigmented guinea pigs (250–400 g) of either sex were anesthetized (urethane, 1.5 g/kg, i.p.) and tracheotomized but were allowed to breath unassisted. ECG and rectal temperature were monitored throughout each experiment, and temperature was maintained at 38° ± 1° C. Additional urethane was administered as required to maintain an adequate depth of anesthesia.

Surgical procedures have been described previously (Kujawa et al., 1994). Briefly, cartilaginous ear canals were exposed and partially removed to allow optimum coupling of the sound delivery systems to the two ears. A subgroup of these animals was used in the cochlear perfusion experiments described in this report. Thus, in all animals, the right auditory bulla was exposed and opened ventrally to gain access to the cochlea and tendons of the right middle ear muscles were sectioned.

2.2. Stimulus generation and response monitoring

The DPOAE under primary investigation during continuous ipsilateral stimulation was the f_2-f_1 DPOAE at 1.25 kHz. For certain ipsilateral and all contralateral stimulus conditions, we also monitored the cubic distortion product at the $2f_1-f_2$ frequency (5

kHz). The instrumentation employed in these experiments has been described (Kujawa et al., 1994). Briefly, responses were elicited by equilevel primary stimuli ($f_1 = 6.25$ kHz; $f_2 = 7.5$ kHz) generated by oscillators, routed through attenuators to separate speakers and delivered to the right ear of each animal by an acoustic probe assembly. Output from the probe microphone was led, via a microphone preamplifier, to a dynamic signal analyzer for averaging (10 discrete spectra) and display (span = 1 kHz; CF = DP frequency; BW = 3.75 Hz). The noise floors associated with these display windows averaged approximately −15 dB SPL for the f_2–f_1 DPOAE and −18 dB SPL for the $2f_1$–f_2 DPOAE when measured at points ±50 Hz of the distortion product frequency. In some experiments, distortion products (f_2–f_1 and $2f_1$–f_2) to other primary pairs were generated; details pertaining to those stimulus conditions will be discussed where appropriate. For contralateral suppression measures, the contralateral stimulus was a 70 dB SPL overall level wideband noise (WBN; 0.9–15.7 kHz), generated and delivered to the left ear as detailed in previous reports (Kujawa et al., 1993; Kujawa et al., 1994).

2.3. Baseline measures

Baseline measures of f_2–f_1 (1.25 kHz) and $2f_1$–f_2 (5 kHz) distortion product growth with increasing stimulus level (25–70 dB SPL in 5 dB steps) were obtained at the right ear of each animal following surgical exposure of the cochlea and middle ear muscle section. Only those animals whose DPOAE growth functions conformed to laboratory norms following these procedures participated in the experiments that followed. A 15 min period without stimulation separated these measures from the next period of primary stimulation.

The influence of continuous, moderate-level (60 dB SPL) primary stimulation on the f_2–f_1 DPOAE at 1.25 kHz was studied in all animals ($N = 35$). The following stimulation and response monitoring protocol was employed: 100 consecutive 10-spectra averages of distortion product amplitude were obtained during continuous primary stimulation. Each of these averages required approximately 5 s to complete for a total of 500 s (8.3 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 10-spectra averages of distortion product amplitude were obtained (total time approximately 200 s or 2.3 min of stimulation). Here again, and for all subsequent stimulus manipulations, a 15 min period of rest from primary stimulation separated each test condition from the next.

Contralateral suppression of both the f_2–f_1 and $2f_1$–f_2 DPOAEs also was studied in all animals. Methods were similar to those employed previously (Kujawa et al., 1994). Briefly, 5 consecutive, 10-spectra averages of DPOAE amplitude (to equilevel primaries at 60 dB SPL) were obtained in the absence of contralateral stimulation. The WBN was then introduced to the contralateral ear and 5 consecutive, 10-spectra averages of distortion product amplitude were obtained during contralateral stimulation. The noise was removed and 5 additional 10-spectra averages of DPOAE amplitude were obtained.

Following these baseline response characterizations, animals were employed in experiments designed to characterize the dependence of these amplitude alterations on the stimulus variables of intensity, duration and frequency and on DPOAE type and/or they served as subjects in the cochlear perfusion or nerve section studies.

2.4. Response characterization experiments

Intensity effects

To investigate the influence of primary level on f_2–f_1 DPOAE amplitude alterations, a subgroup of animals was tested with equilevel primaries at 40, 50 and 70 as well as 60 dB SPL. Responses were monitored as described above.

Duration effects

To clarify the dependence of these time-varying changes in f_2–f_1 DPOAE amplitude on continuous primary stimulation, f_2–f_1 amplitude changes associated with intermittent periods of short-duration (5 s) primary stimulation ($L_1 = L_2 = 50$–70 dB SPL) were studied over the same time period. For these comparisons, single 10-spectra averages were obtained at times corresponding to the first average of the continuous series, the 100th average and the final (40th) average following the return to stimulation. Alternatively, following the initial period of continuous primary stimulation (500 s) and 1 min rest, f_2–f_1 distortion product recovery was tracked using 5 s periods of stimulation obtained at 30 s intervals. Results were compared to those obtained in the same animals using our standard (baseline) protocol.

The influence of rest period duration on amplitude changes in the f_2–f_1 DPOAE as recorded following the first period of continuous stimulation was investigated using primaries at 60 dB SPL. This was accomplished by halving (30 s) or doubling (2 min) the period of rest from continuous primary stimulation and then comparing results to those obtained in the same animals with the standard (1 min) rest.

Frequency effects

The influence of primary frequency was investigated by comparing alterations in the amplitude of the f_2–f_1 DPOAE when generated by lower ($f_1 = 2.5$ kHz; $f_2 = 3$

kHz) and by higher ($f_1 = 10$ kHz; $f_2 = 12$ kHz) frequency primaries to results obtained with the standard primary pair ($f_1 = 6.25$ kHz; $f_2 = 7.5$ kHz). For these measurements, the f_2/f_1 ratio was held constant at 1.2 and primaries were equal in level at 60 dB SPL.

DPOAE type

The effect of DPOAE type on the magnitude and time course of the amplitude alterations during continuous primary stimulation was investigated under several separate conditions: First, amplitude alterations in the cubic ($2f_1-f_2$) DPOAE at 5 kHz were compared to those recorded for the quadratic (f_2-f_1) DPOAE at 1.25 kHz for identical stimulus frequency and intensity conditions ($f_1 = 6.25$ kHz; $f_2 = 7.5$ kHz; $L_1 = L_2 = 60$ dB SPL). Second, a $2f_1-f_2$ DPOAE was elicited at 1.25 kHz ($f_1 = 1.55$ kHz; $f_2 = 1.86$ kHz; $L_1 = L_2 = 60$ dB SPL) and amplitude alterations observed for this distortion product were compared to those for the f_2-f_1 DPOAE at the same frequency and for the same primary levels. Finally, in an attempt to compare f_2-f_1 and $2f_1-f_2$ DPOAEs similar in baseline amplitude, the $2f_1-f_2$ DPOAE at 5 kHz was elicited by equilevel primaries at 50 dB SPL and response alterations to continuous stimulation under these conditions were compared to those obtained for the f_2-f_1 DPOAE response to higher level (60 dB SPL) primaries.

2.5. Cochlear perfusion experiments

Perfusion experiments were undertaken using methods described previously (Kujawa et al., 1994). The artificial perilymph had a composition of (in mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; NaH_2PO_4, 1; $MgCl_2$, 1; glucose, 11; $NaHCO_3$, 12 with a resulting pH of 7.4 when brought into solution in a deionized and filtered water base. Experimental drugs tested for their effects on ipsilateral and contralateral stimulation-related DPOAE amplitude alterations were the following: bicuculline (bicuculline methiodide, 10 μM, Sigma), curare (d-tubocurarine chloride, 1 μM, Sigma) and TTX (tetrodotoxin, 1 μM, Sigma). The agents were dissolved in the artificial perilymph (pH 7.4). Perfusates were introduced into the cochlear perilymph at a rate of 2.5 μl/min for 10 min through a hole in basal turn scala tympani and were allowed to flow from the cochlea through an effluent hole placed in basal turn scala vestibuli. Effluent was absorbed within the bulla using small cotton wicks.

For the purposes of the perfusion experiments, animals were divided into 4 groups: One group of animals ($N = 5$) received 8 consecutive perfusions of the control (artificial perilymph) solution alone. Effects of the OC efferent antagonists, bicuculline and curare, were tested in another group of animals ($N = 5$). These animals first received 2 perfusions of the control (artificial perilymph) solution alone. These perfusions were followed, in order, by a single perfusion of bicuculline, 2 artificial perilymph washes, a single perfusion of curare and two additional artificial perilymph washes. To insure that drugs were not being diluted with replacement CSF during the long response monitoring periods post-perfusion, a third group of animals ($N = 4$) received these drugs after first undergoing blockade of the cochlear aqueduct as described by Jenison et al. (1985). Effects of TTX were studied in a fourth group of animals ($N = 6$). Again, the first 2 perfusions were of artificial perilymph alone. These perfusions were followed by a single perfusion of TTX followed by 2 artificial perilymph washes. Immediately following each perfusion, measures of f_2-f_1 and $2f_1-f_2$ DPOAE amplitudes before, during and after contralateral WBN stimulation were obtained followed by measures of f_2-f_1 amplitude during continuous ipsilateral stimulation (60 dB SPL) as described for baseline characterizations.

2.6. OC nerve section experiments

The effect of OC section at the brainstem midline on the ipsilateral stimulation-related changes in f_2-f_1 amplitude was studied in $N = 4$ animals. Such a section should effectively remove input from the vast majority of ipsilaterally-responsive (i.e., crossed) MOC neurons to each cochlea (Liberman and Brown, 1986). In these animals, the middle cerebellar vermis was aspirated, revealing the floor of the IVth ventricle. Measures of ipsilateral stimulation-related effects on f_2-f_1 DPOAE amplitude to 60 dB SPL primaries were obtained to identify changes in response characteristics that might be associated with these surgical manipulations. A midline cut spanning the anterior-posterior extent of the exposed IVth ventricle floor at a depth of approximately 2 mm was then made and ipsilateral and contralateral stimulation measures were obtained. Results of both sets of measures were compared to baseline data for these animals.

Effects of treatments on ipsilateral and contralateral stimulation-related changes in DPOAE amplitude were quantified using repeated measures analysis of variance (ANOVA) and Tukey post-hoc tests. The care and use of the animals reported on in this study were approved by LSUMC's Institutional Animal Care and Use Committee.

3. Results

3.1. Amplitude alterations during continuous ipsilateral stimulation

During continuous stimulation with moderate-level (60 dB SPL) primaries, the f_2-f_1 DPOAE was ob-

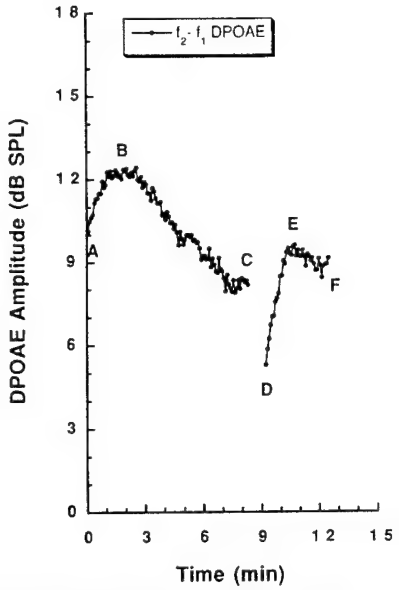

Fig. 1. Effect of continuous primary stimulation on f_2-f_1 DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Each data point represents a 10 spectra average and required 5 s to complete. Break in response amplitude trace (C–D) represents 1 min with no primary stimulation. Points A–F thus identified in each trace were used to calculate magnitudes of component amplitude changes, slopes of suppression and recovery functions and for statistical analyses across animals.

served to undergo stereotyped amplitude alterations. A representative example is shown in Fig. 1. Following a short period of amplitude growth during the first 2 min of continuous primary stimulation, the f_2-f_1 DPOAE underwent a slow decline which either progressed at a similar rate throughout the remainder of the stimulation period (approximately 6–7 min) as in this example, or which gradually fell to a new, lower level and then changed comparatively little during the last few minutes of stimulation. Following a 1 min rest from continuous primary stimulation, a return to stimulation found the DPOAE further suppressed from its pre-rest amplitude. Thereafter, f_2-f_1 amplitude increased rapidly, again reaching a maximum approximately 2 min into the period of continuous primary stimulation. Periodic checks of primary amplitudes revealed changes less than ± 0.1 dB across similar periods of stimulation.

This general response configuration was obtained for 33 of 35 animals tested in this series. In the remaining 2 animals, the post-rest amplitude suppression was absent, but all other amplitude alterations followed the characteristic pattern. Six points (A–F) were identified on each response amplitude function (see Fig. 1). These values were used to calculate the magnitudes of the various amplitude changes, the overall slopes of the suppression and recovery functions and for statistical analyses. The period of amplitude growth observed following stimulus onset will be referred to as an 'on-effect' of continuous primary stimulation. The first such on-effect is represented as the change in amplitude from point A (DPOAE amplitude at the first 10 spectra average) to point B (the 10 spectra average yielding the maximum DPOAE amplitude during the first period of continuous stimulation). In the 35 animals tested with equilevel primaries at 60 dB SPL, this amplitude increase averaged 1.52 ± 0.14 dB at its peak (B), which occurred 1 min 44 s ± 6 s into the period of continuous primary stimulation (A to B slope = 0.88 dB/min). After reaching this amplitude maximum, the response declined slowly during the remainder of the stimulation period (B to C slope = -0.42 dB/min). At the end of this period of continuous primary stimulation (C), the amplitude of the distortion product was reduced 1.24 ± 0.31 dB from its amplitude at onset (A). However, it was reduced from its peak value (B) by 2.75 ± 0.25 dB. At the return to stimulation (D) following a 1 min rest, the f_2-f_1 DPOAE was further suppressed from this pre-rest value (C) by 1.26 ± 0.12 dB. Of interest, the magnitude of the amplitude decline occurring during this 1 min rest from continuous stimulation is 3 times that observed for a comparable time period during which the primaries were delivered continuously. This post-rest amplitude suppression (the change in DPOAE amplitude from point C to point D) will be referred to as the 'off-effect'. Following the return to stimulation, response amplitude increased rapidly (2.44 ± 0.18 dB at point E). This second on-effect (ΔD–E) demonstrated a time course similar to the first, reaching a new maximum 2 min 2 s ± 7 s into the second period of continuous stimulation (D to E slope = 1.20 dB/min). After reaching this amplitude maximum, the response again began on a downward course, clearly evident at point F (E to F slope = -0.43 dB/min). All amplitude changes from baseline reached significance ($P < 0.01$).

When monitored over repeated trials in the same animal, these characteristic f_2-f_1 amplitude alterations demonstrated little variability. Fig. 2 displays the results of repeated averages separated by periods of

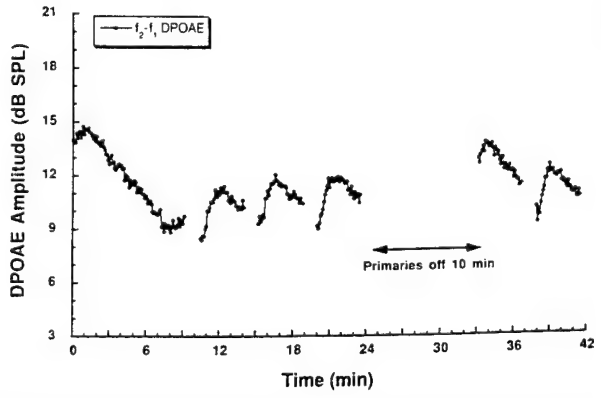

Fig. 2. Effect of repeated primary stimulations separated by 1 min or 10 min rest periods on f_2-f_1 DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL).

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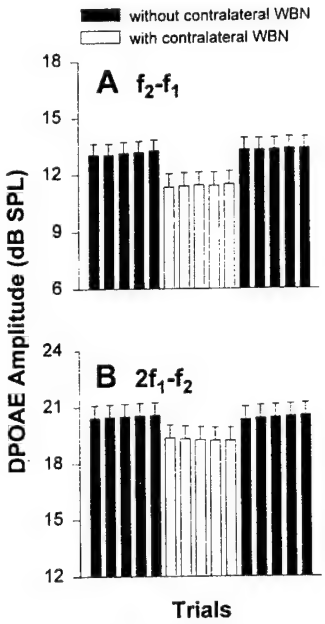

Fig. 3. Effect of contralateral WBN on (A) f_2-f_1 and (B) $2f_1-f_2$ DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). DPOAEs were monitored before (5 trials, solid bars), during (5 trials, open bars) and after (5 trials, solid bars) presentation of WBN (70 dB SPL) to the contralateral ear. Each 'trial' represents a 10 spectra average and required 5 sec to complete. Data are represented as means ± S.E. (N = 35).

rest (1 min; 10 min) for one representative animal. Although the extent of the amplitude decline is limited in subsequent trials (due to the shortened periods of continuous stimulation; approximately 3.3 vs 8.3 min), on- and off-effects are nearly identical in magnitude and time course and the slopes of the growth and decay portions of the curves remain constant. After a longer period of rest from continuous stimulation (10 min), some additional recovery of distortion product amplitude is evident. Similar results were obtained in 4 additional animals.

3.2. Amplitude alterations during contralateral WBN stimulation

Contralateral suppression of both f_2-f_1 and $2f_1-f_2$ DPOAEs was observed in 34 of 35 animals tested. The f_2-f_1 DPOAE was reduced approximately 1.7 dB (Fig. 3A) and the $2f_1-f_2$ DPOAE was reduced approximately 1.2 dB (Fig. 3B) in the presence of a 70 dB SPL contralaterally-presented WBN. In both cases, the contralateral stimulation-associated reductions in DPOAE amplitude reached significance ($P < 0.001$). When contralateral sound effects are expressed in terms of percent reduction of baseline distortion product amplitude, the f_2-f_1 DPOAE was reduced by roughly 13%, the $2f_1-f_2$ DPOAE by 6%.

3.3. Effects of stimulus variables and DPOAE type

Intensity effects

On average ($N = 9$), the magnitudes of the component amplitude alterations associated with continuous ipsilateral stimulation decreased as stimulus intensity was increased (Figs. 4A–C). Intensity-related changes in on- (ΔA–B; ΔD–E) and off-effect magnitudes (ΔC–D) reached overall significance, with post-hoc comparisons (Tukey) revealing significant differences between values obtained at 50 and 60 dB SPL ($P < 0.01$) and 50 and 70 dB SPL ($P < 0.01$) but not 60 and 70 dB SPL ($P > 0.05$). The magnitude of the pre-rest amplitude decline (ΔB–C) associated with 50 dB SPL primaries was more variable between animals. Thus, intensity-related alterations in the magnitude of pre-rest amplitude decline failed to reach overall significance ($P > 0.05$). Responses to primary stimuli at 40 dB SPL ($N = 3$ animals) were very small and were more variable in amplitude over repeated averages within animals. It could not, therefore, reliably be determined whether effects of continuous stimulation continued to grow as primary level was reduced further.

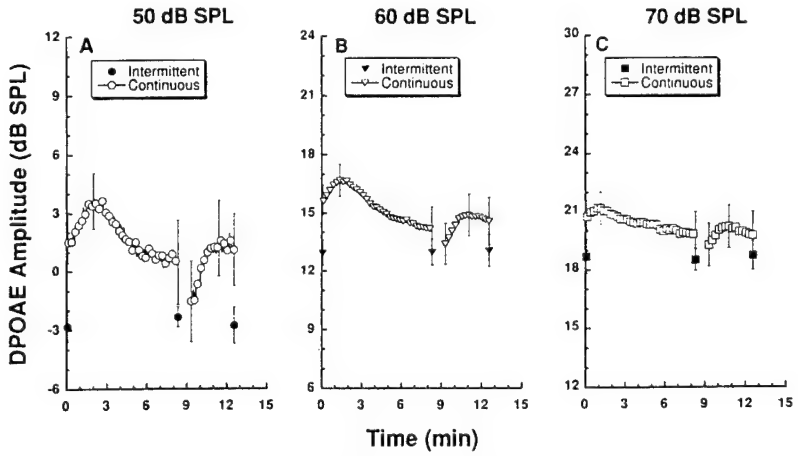

Fig. 4. Effect of primary intensity on f_2-f_1 DPOAE amplitude alterations ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 50$–70 dB SPL) associated with continuous (N = 9) and intermittent (N = 6) stimulations. Data are represented as means ± S.E.

Duration effects

In a subgroup of these animals ($N = 6$), DPOAE amplitudes to 50, 60 or 70 dB SPL primaries presented intermittently (at times corresponding to points A, C and F) remained very stable (Figs. 4A–C). On average, response amplitude to 60 dB SPL primaries varied less than 0.1 dB over a corresponding time period. We did not study how closely-spaced in time the periods of primary stimulation must be in order to produce these characteristic amplitude alterations. However, for periods of intermittent primary stimulation separated by 30 s intervals, distortion product recovery from the post-rest suppression followed a gradual course over the entire period of post-rest monitoring and the degree of amplitude recovery was more variable than that observed using the standard, continuous stimulation protocol.

Both the magnitude of the off-effect and the time course of the subsequent recovery were sensitive to the duration of rest from continuous stimulation. In general, the magnitude of the off-effect associated with either 30 s or 2 min periods of rest was < 0.5 dB. Moreover, although the times to maximum amplitude following the 1 and 2 min rests were similar, this time course was variable across animals following the abbreviated (30 s) rest.

Frequency effects

For these experiments, ($N = 5$) two additional frequency pairs were employed: $f_1 = 2.5$ kHz and $f_2 = 3$ kHz which yielded an f_2–f_1 DPOAE at 500 Hz and $f_1 = 10$ kHz and $f_2 = 12$ kHz which yielded an f_2–f_1 DPOAE at 2 kHz. The magnitudes of the amplitude alterations in the resulting f_2–f_1 DPOAEs were then compared to those observed with the standard primary pair ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz; f_2–$f_1 = 1.25$ kHz). No clear relationship to frequency emerged across the primary frequency range tested: In some animals, f_2–f_1 amplitude alterations were similar in magnitude for the 3 primary pairs tested and in some animals effects were larger when distortion products were elicited by mid- or high- but never low-frequency primaries.

DPOAE type

Under identical conditions of stimulation, amplitude alterations in the $2f_1$–f_2 DPOAE at 5 kHz were substantially smaller than those seen for the f_2–f_1 DPOAE at 1.25 kHz. In general, $2f_1$–f_2 amplitude increased slightly (< 1 dB) over the first few minutes of continuous primary stimulation and subsequently maintained this higher amplitude for the remainder of the stimulation period (Fig. 5). In none of these animals did we observe the continuous stimulation-related decline in $2f_1$–f_2 DPOAE amplitude observed for the f_2–f_1 DPOAE. In most animals, a small (approximately 0.5 dB), suppressive off-effect was evident. Our ability to observe amplitude alterations in this distortion product was not improved when stimulus intensity was reduced to 50 dB SPL, nor was it improved when $2f_1$–f_2 frequency was matched with that of the f_2–f_1 DPOAE under primary investigation in these experiments (1.25 kHz). In these latter groups of animals, however, on- and off-effects were occasionally reversed in sign – that is, on-effects appeared as small reductions in the amplitude of the $2f_1$–f_2 DPOAE and off-effects as small increases in response amplitude.

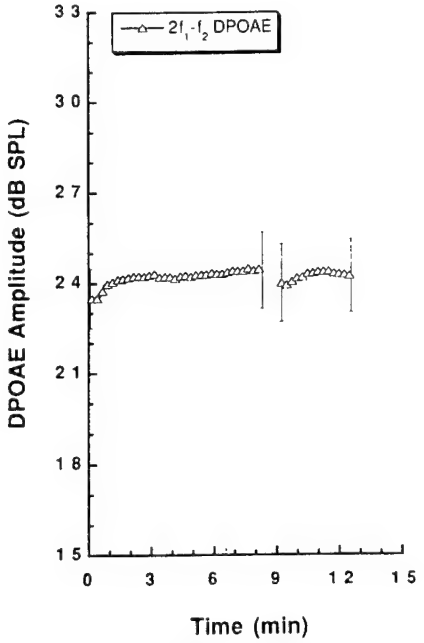

Fig. 5. Effect of continuous primary stimulation on $2f_1$–f_2 DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Data are represented as means ± S.E. across N = 4 animals.

3.4. Pharmacologic blockade of OC efferents

Control perfusions

In contrast to the remarkable stability of the $2f_1$–f_2 DPOAE to the manipulations associated with cochlear perfusion (e.g., Kujawa et al., 1993; Kujawa et al., 1994), the f_2–f_1 DPOAE was altered substantially by perfusion. All components of the perstimulus f_2–f_1 response alterations were enhanced by the first perfusion of the control solution (artificial perilymph; AP). In particular, the pre-rest amplitude suppression (ΔA–C) increased from an average of less than 3 dB pre-perfusion to roughly 8 dB post-AP #1. Similar enhancement of response suppression was not observed when primaries were presented only intermittently over the same time period, suggesting that the additional amplitude decline is not due solely to the long monitoring periods required following perfusion. Response alterations stabilized, however, following the initial AP perfusion and additional control perfusions ($N = 7$) in

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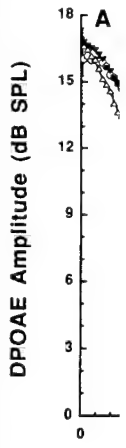

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sions (W2b).
point; connec

each of 5 animals produced no further, significant changes in any response parameter. Thus, for each animal, values obtained following AP perfusion #2 were employed as the new post-perfusion baselines to which drug-related changes were compared. For all post-perfusion measures (control and experimental), contralateral suppression studies preceded immediately our monitoring of ipsilateral stimulation-related changes in f_2–f_1 amplitude. As a result of the prior stimulations, the initial on-effect (ΔA–B) described for the pre-perfusion measures is not observed in these post-perfusion amplitude records. The on-effect represented by the change in f_2–f_1 amplitude from points D–E remains, however, and was employed in statistical analyses of drug effects.

Drug perfusions

Intracochlear perfusion of bicuculline (10 μM) reduced, but did not block the component alterations in f_2–f_1 amplitude during ipsilateral stimulation (Fig. 6A). None of these drug-related changes reached significance ($P > 0.05$). Curare (Fig. 6B), at an order of magnitude lower concentration (1 μM), reduced significantly the pre-rest amplitude decline ($P < 0.05$), but drug-related changes in on- and off-effects failed to reach significance. In contrast, contralateral WBN effects on f_2–f_1 and $2f_1$–f_2 DPOAEs were blocked reversibly by both drugs (Figs. 7A–B), consistent with our previous findings for the $2f_1$–f_2 DPOAE (Kujawa et al., 1994). Also consistent with our previous findings, the amplitude of the $2f_1$–f_2 DPOAE, recorded in the absence of contralateral stimulation, was increased

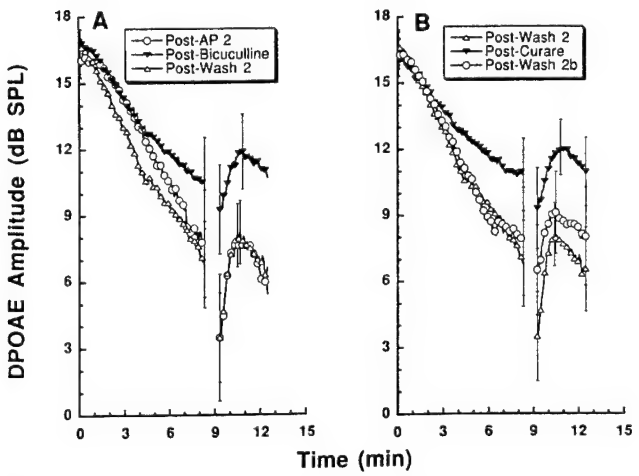

Fig. 6. Effect of OC efferent antagonists on f_2–f_1 DPOAE amplitude alterations during continuous primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). (A) Response amplitude (means ± S.E.; N = 5) as recorded following the second control perfusion (AP2), bicuculline (10 μM) and the second wash perfusion (W2). (B) Response amplitude (means ± S.E.; N = 5) as recorded following W2 above, curare (1 μM) and two additional wash perfusions (W2b). For reading ease, symbols occur only at every 3rd data point; connecting lines follow all data points.

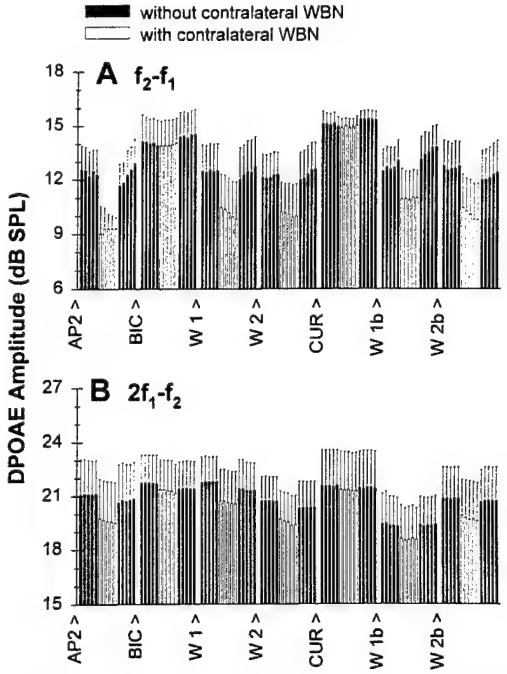

Fig. 7. Effect of OC efferent antagonists on contralateral WBN suppression of (A) f_2–f_1 and (B) $2f_1$–f_2 DPOAEs ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). In each panel, DPOAE amplitudes (means ± S.E., N = 5) are shown following perfusions of control solutions (AP2), bicuculline (10 μM, BIC), wash perfusions (W1, W2), curare (1 μM, CUR) and two additional washes (W1b, W2b).

from levels observed following perfusions of artificial perilymph. We now extend those observations to include similar effects of these antagonists on f_2–f_1 DPOAE amplitude.

In view of the long post-perfusion monitoring periods employed in these studies, it appeared possible that the inability of the drugs to block the ipsilateral effects might relate to a gradual washing of the drugs from the cochlear perilymph with replacement CSF before post-perfusion measurements could be completed. In pilot experiments, post-drug contralateral suppression was monitored after, rather than before ipsilateral stimulation measures and, in those animals, contralateral suppression could not be observed, suggesting that the drugs remained effective in blocking the efferents even at this extended time post-perfusion. Nevertheless, an additional subgroup of animals ($N = 4$) was tested in which the cochlear aqueduct was blocked prior to perfusion. In these animals, the magnitude of blockade of the ipsilateral effects was not altered substantially from that seen in aqueduct-patent animals. Of interest, when aqueduct-patent animals were subsequently anesthetized with Nembutal (30 mg/kg, i.p.) and ipsilateral stimulation measures repeated, reductions in the magnitudes of the component f_2–f_1 amplitude alterations were similar to those obtained following antagonist perfusions in urethane-anesthetized animals (see Fig. 8).

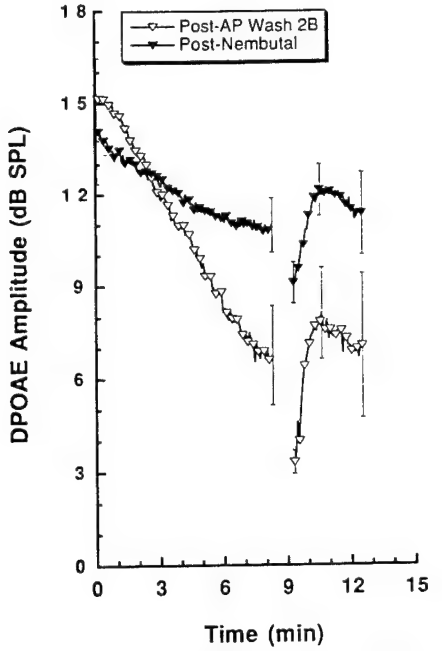

Fig. 8. Effect of Nembutal anesthesia on f_2-f_1 DPOAE amplitude alterations during continuous primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Perfusion experiments completed under urethane anesthesia (AP Wash 2B) were followed by administration of Nembutal (30 mg/kg, i.p.) to N = 3 animals. Approximately 15 min later, post-Nembutal response measures were obtained. Symbols occur only at every 3rd data point; connecting lines follow all data points.

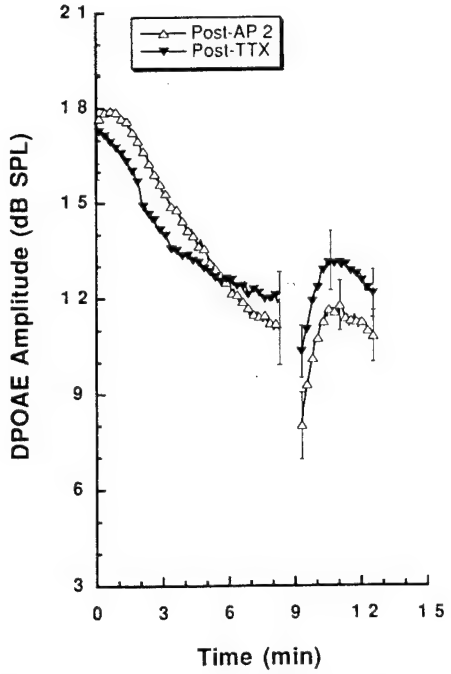

Fig. 9. Effect of TTX on f_2-f_1 DPOAE amplitude alterations during continuous primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Shown are response amplitude means ± S.E. (N = 6) following perfusions of control solutions (AP2) and TTX (1 μM). Symbols occur only at every 3rd data point; connecting lines follow all data points.

The concentrations of OC antagonists employed in the perfusion experiments were chosen based on detailed studies of dose-response relations for the pharmacologic blockade of contralateral sound suppression (Kujawa et al., 1994). In those experiments, bicuculline (10 μM) and curare (1 μM) both were effective in blocking that efferent-mediated response. The substantially lesser potency of these drugs in blocking the ipsilateral effects under study here suggested that further clarification of the extent of efferent involvement was necessary. Thus, two additional manipulations were performed in separate groups of animals. In one group ($N = 6$), the cochlear perilymph was perfused with TTX (1 μM) which should block all action potential-mediated activity. Following such perfusions, the magnitude of the off-effect was reduced (Fig. 9), but the pre-rest amplitude decline did not differ significantly from baseline values ($P > 0.05$). In these same animals, contralateral suppression of both distortion products (Figs. 10A–B) was prevented ($P < 0.01$) and the round window-recorded auditory nerve compound action potential (CAP) at 10 kHz was not observed at any intensity (to 102 dB SPL) following perfusion of the cochlear perilymph with TTX. Figs. 10A-B also reveal that the absolute amplitudes of the f_2-f_1 and $2f_1-f_2$ DPOAEs were affected differently by this manipulation: The $2f_1-f_2$ DPOAE is reduced in amplitude

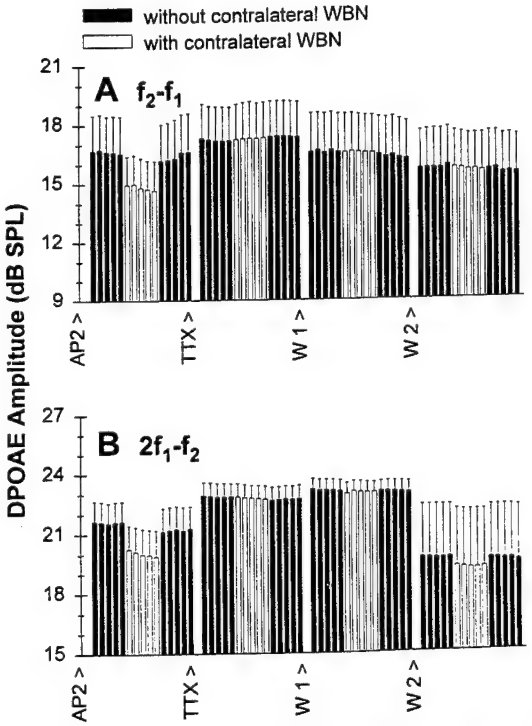

Fig. 10. Effect of TTX on contralateral WBN suppression of (A) f_2-f_1 and (B) $2f_1-f_2$ DPOAEs ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). In each panel, DPOAE amplitudes (means ± S.E., N = 6) are shown following perfusions of control solutions (AP2), TTX (1 μM) and two wash perfusions (W1, W2).

following attempts at washout; f_2–f_1 amplitude is little changed.

3.5. OC efferent section

A second manipulation performed to clarify the extent of OC involvement in these ipsilateral effects involved section of OC neurons at the midline of the floor of the IVth ventricle. The overall amplitude of the f_2–f_1 DPOAE was reduced following surgical exposure of the floor of the IVth ventricle, but the magnitudes of the component amplitude alterations in this distortion product were essentially unchanged (Fig. 11). Section of the OC efferents at the midline of the IVth ventricle floor produced no further changes in absolute DPOAE amplitude and did not alter substantially the f_2–f_1 amplitude alterations observed during continuous ipsilateral stimulation. In contrast, contralateral suppression of both f_2–f_1 and $2f_1$–f_2 DPOAEs was prevented by midline section (Figs. 12A-B). This may have occurred due to involvement of UMOC neurons in the midline cuts, as these fibers can course very near the brainstem midline, at least in cat (Gifford and Guinan, 1987) and mouse (Brown, 1993b). Here again, f_2–f_1 and $2f_1$–f_2 distortion components display different post-manipulation behaviors: The reduction in absolute distortion product amplitude seen for the f_2–f_1 DPOAE following exposure of the IVth ventricle is not reflected in the $2f_1$–f_2 DPOAE.

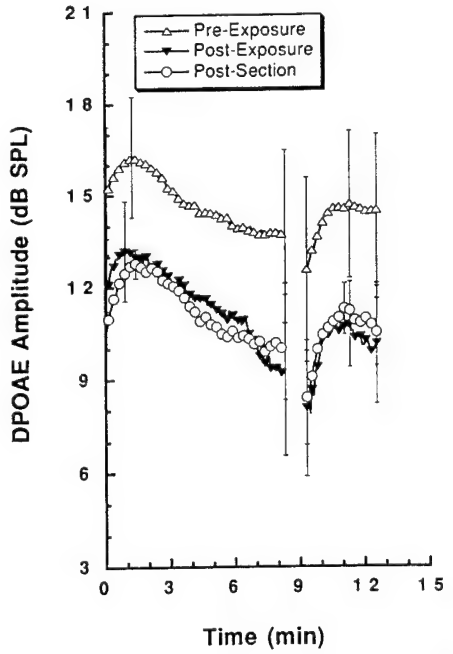

Fig. 11. Effect of OC section on f_2-f_1 DPOAE amplitude alterations during continuous primary stimulation (f_1 = 6.25 kHz, f_2 = 7.5 kHz, $L_1 = L_2$ = 60 dB SPL). Shown are response amplitude means ± S.E. (N = 4) prior to exposure of the IVth ventricle (Pre-Exposure), following IVth ventricle exposure (Post-Exposure) and following midline section of OC fibers (Post-Section). Symbols occur only at every 3rd data point; connecting lines follow all data points.

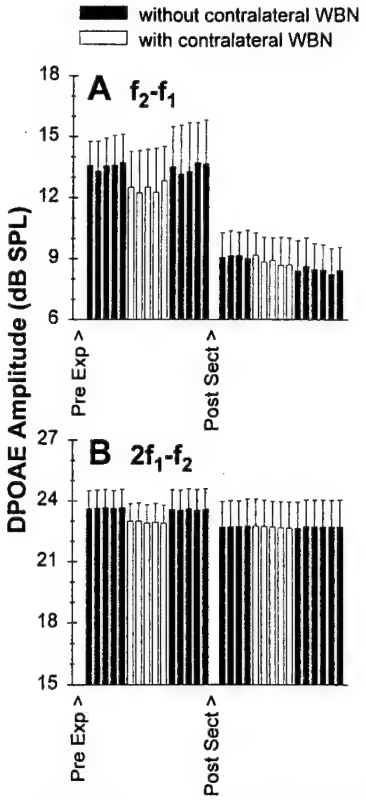

Fig. 12. Effect of OC section on contralateral WBN suppression of (A) f_2-f_1 and (B) $2f_1-f_2$ DPOAEs (f_1 = 6.25 kHz, f_2 = 7.5 kHz, $L_1 = L_2$ = 60 dB SPL). In each panel, DPOAE amplitudes (means ± S.E., N = 4) are shown before exposure of the IVth ventricle (Pre-Exp) and following midline OC section (Post Section).

4. Discussion

4.1. Alterations in magnitude of auditory system response

Alterations in the magnitude of auditory system response during and following continuous stimulation are well documented. Alterations with short (ms) time courses have been observed in responses of afferent neurons (e.g., Kiang et al., 1965; Young and Sachs, 1973a) and with longer (min) time courses in behavioral responses to sound (e.g., Moore, 1968; Noffsinger and Tillman, 1970; Young and Sachs, 1973b). Response enhancements at the onset of stimulation, perstimulatory declines and post-stimulatory depressions in response magnitude all have been observed. Of importance here, such alterations have been observed for stimulations at intensity levels that should not produce permanent, deleterious effects on cochlear structures.

4.2. f2–f1 amplitude alterations

Given these observations, it should not be surprising that time-varying alterations in the amplitude of the f_2–f_1 DPOAE are observed during continuous primary stimulation. The perstimulatory amplitude changes reported here are qualitatively similar to those reported by others for similar conditions of stimulation. Both Brown (1988) and Whitehead et al. (1991) reported initial increases in f_2–f_1 DPOAE amplitude that were followed by gradual declines during continued primary stimulation. Consistent with results presented by Whitehead and colleagues, we observed the response amplitude maximum (on-effect) to occur approximately 2 min into the period of continuous stimulation. Thereafter, response amplitude declined gradually over the remaining period of stimulation. Although the magnitude of this amplitude decline was similar across animals for 60 dB SPL primaries, it became substantially more variable as primary level was reduced. Brown (1988), employing lower primary levels (20–55 dB SPL), also reported large variations in magnitude of the f_2–f_1 amplitude decline. This variability may account, at least in part, for the reported differences in magnitude of effect between the various investigations of this phenomenon.

A suppressive off-effect of continuous primary stimulation has not been reported by previous investigators of these f_2–f_1 amplitude alterations. In reviewing those reports, it was noted that rest periods from primary stimulation employed in those studies generally exceeded the 1 min rest employed here. Furthermore, when the distortion product was monitored by Brown (1988) after a 3 min rest, partial recovery of response amplitude already was apparent. In the present experiments, the magnitude of this off-effect was substantially reduced when rest periods of either 30 s or 2 min were employed. Thus, it is not surprising that such additional response suppression was not observed by Whitehead et al. (1991) who employed rest periods of 5 min or longer and Brown (1988) who generally employed rest periods exceeding 2–3 min. According to the present results, the lengths of rest used by these investigators would likely have precluded their observation of this time-dependent effect.

4.3. 2f1–f2 amplitude alterations

Consistent with earlier investigations of these perstimulatory DPOAE amplitude changes, corresponding changes in the amplitude of the $2f_1$–f_2 DPOAE were very small or were not observed. In psychophysical paradigms, quadratic (f_2–f_1) and cubic ($2f_1$–f_2) nonlinearities can display different behaviors (Goldstein, 1967; Zwicker, 1979). Moreover, these two distortion components can exhibit different vulnerabilities to cochlear insult (Kujawa and Bobbin, unpublished observations; see also Figs. 10 and 12 in the present report). Such observations suggest that the mechanisms underlying generation of cubic and quadratic cochlear nonlinearities are not identical (for discussion, see Brown, 1993a).

4.4. Contralateral suppression

Although Kirk and Johnstone (1993) failed to observe convincing alterations in $2f_1$–f_2 amplitude during periods of contralateral WBN stimulation, results obtained in the present experiments are in excellent agreement with previous reports of $2f_1$–f_2 amplitude suppression by contralateral noise (Kujawa et al., 1993; Kujawa et al., 1994; Puel and Rebillard, 1990; Puria et al., 1992). Consistent with the report of Kirk and Johnstone, we also observed contralateral sound suppression of the f_2–f_1 DPOAE in virtually all animals tested. On average, the magnitude of this suppression was slightly greater than that observed for the $2f_1$–f_2 DPOAE although, in individual animals, it could be substantially greater.

4.5. Sites and mechanisms underlying response modulation

One possible mechanism that might account for the f_2–f_1 amplitude changes observed during continuous ipsilateral stimulation involves modulation of the cochlear mechanical response by OC neurons. This efferent influence would be accomplished via neurotransmitter (and neuromodulator) substances. Based on the finding of bicuculline (10 μM) blockade of ipsilateral and contralateral stimulation-related alterations in f_2–f_1 amplitude, Kirk and Johnstone (1993) suggested that both processes are controlled via GABAergic efferent pathways. In the present experiments, however, the GABA antagonist, bicuculline, at a concentration of 10 μM, was less effective than the nicotinic cholinergic antagonist, curare, at 1 μM, in antagonizing the perstimulatory amplitude reductions in f_2–f_1. Neither drug blocked these ipsilateral effects completely yet both drugs blocked completely contralateral sound suppression of f_2–f_1 and $2f_1$–f_2 DPOAEs. Our ability to pharmacologically antagonize these ipsilateral effects was not improved substantially in animals in which the cochlear aqueduct had been blocked prior to perfusion. Finally, neither TTX nor OC section altered substantially the ipsilateral stimulation-related effects.

We have previously characterized the pharmacology of contralateral sound suppression of the $2f_1$–f_2 DPOAE response to primaries within the range studied by Kirk and Johnstone (Kujawa et al., 1994). Results of those studies suggested that a nicotinic-like

cholinergic receptor mediated such suppression. Specifically, nicotinic antagonists (α- and κ-bungarotoxins, curare) and strychnine were most potent (IC_{50} values were achieved at nanomolar concentrations of these drugs). Bicuculline also was surprisingly effective in blocking this response suppression ($IC_{50} = 2.39 \times 10^{-6}$). Nicotinic receptor sensitivity to blockade by both strychnine and bicuculline has been demonstrated in several systems (see Kujawa et al., 1994 for review). This sensitivity has been suggested to reflect a strong structural homology between the receptors belonging to the super family that includes receptors for ACh, glycine and GABA (e.g., Grenningloh et al., 1987; Schofield et al., 1987).

At present, we cannot explain the differences between the two investigations with regard to the magnitudes of effect of bicuculline and TTX. The discussion of bicuculline's effects by Kirk and Johnstone (1993), however, suggests that substantial variability between animals in magnitude of drug effect was encountered. Perfusion also increased the variability in the f_2–f_1 DPOAE in our own experiments – both for control and experimental solutions. Additional procedural differences between the investigations may have contributed to observed differences in drug effects, as well. In the present experiments, middle ear muscles were sectioned; in the Kirk and Johnstone studies, animals were paralyzed in an attempt to prevent middle ear muscle contraction. There are differences in anesthetic agents employed and in placement of perfusion and effluent holes in the cochlea between the two investigations.

In view of the lack of effect of TTX and OC section on these response alterations, we also cannot explain the partial blockade of these amplitude alterations by the OC antagonists. However, Nembutal produced a blockade of the ipsilateral effects indistinguishable from that associated with bicuculline and curare. This effect certainly cannot be due to selective or specific blockade of receptors for the OC neurotransmitter. Rather, this finding suggests that, at these concentrations, bicuculline, curare and Nembutal may be acting nonselectively to block a channel operating in the OHCs. Evidence for channel blockade by bicuculline and curare was provided at the level of isolated OHCs by Erostegui et al. (1994).

4.6. Does efferent control underlie f_2–f_1 DPOAE amplitude alterations?

Results of the present experiments do not make it possible to specify the mechanism(s) underlying the perstimulatory alterations in f_2–f_1 amplitude described here. The results suggest, however, that they are not primarily the result of efferent control. Moreover, as reviewed by Young and Sachs (1973a), since sound-induced increases in efferent firing rate are maximum at stimulus onset (Fex, 1962) and OC inhibition of afferent activity decreases with time during a maintained stimulus (Wiederhold and Kiang, 1970), it follows that efferent activation should be associated with a perstimulatory response suppression which is maximum near stimulus onset, rather than the observed enhancement of suppression with time. The experiments cannot rule out an entirely peripheral efferent effect on f_2–f_1 DPOAE amplitude; however, if such an influence exists, it does not appear to involve action potential-mediated activity. Additionally, it would have to follow a time course much longer than that described to date for OC-mediated effects – even the recently-described 'slow effects' of OC stimulation (Sridhar et al., 1994) which demonstrated a pharmacology consistent with that previously described for contralateral suppression (Kujawa et al., 1994). A second mechanism that might underlie these ipsilateral effects could involve local, adaptive changes occurring at the level of the hair cells. Adaptation of the hair cell transduction current during sustained mechanical deflection has been demonstrated in vertebrate (although not yet mammalian) hair cells (e.g., Crawford et al., 1989; Eatock et al., 1987; Assad and Corey, 1992). Further, it has been suggested that distortion product responses arise from nonlinearities in hair cell channel gating mechanisms (Jaramillo et al., 1993). It is possible that such adaptive changes could be reflected in otoacoustic emissions. In previous work by Siegel et al. (1982) temporary reductions in the amplitudes of f_2–f_1 and $2f_1$–f_2 distortion components recorded at the level of single afferent units were observed following stimulations as low as 60 dB SPL. The authors noted that these alterations resembled those observed following higher-level, fatiguing stimulations. In subsequent papers, we will report the results of experiments in which we investigated the influence of local, calcium-dependent mechanisms and chronic, moderately-intense noise exposures on these amplitude alterations.

Acknowledgments

The authors are grateful to Ted Glattke for discussions and to Han Wen for programming assistance. This work was supported by NIH DC00722 and DC00007; DAMD 17-93-V-3013, Kam's Fund for Hearing Research and the Louisiana Lions Eye Foundation.

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July 25, 1995 (12:42pm)

Nimodipine, BayK and lowering Ca^{2+} levels in perilymph alter distortion product otoacoustic emissions

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Summary

The distortion product otoacoustic emission (DPOAE), f_2-f_1, displays time-varying amplitude alterations during continuous, primary tone stimulation (T-VAA; Kujawa et al., 1995). The origin of these alterations are unknown. Evidence that efferent neurons contribute little to the changes has been presented (Kujawa et al., 1995; Robertson 1995). The purpose of the present investigation was to examine the hypothesis that these alterations in f_2-f_1 are dependent on Ca^{2+} levels and in particular L-type Ca^{2+} channels. Urethane anesthetized guinea pigs were used. Perilymph spaces of cochleae were alternately perfused with artificial perilymph, artificial perilymph with lowered Ca^{2+} levels, nimodipine, a L-type Ca^{2+} channel antagonist, and BayK, a L-type Ca^{2+} channel activator, at 2.5 μl/min for various times. After each period of perfusion, cubic ($2f_1$-f_2) and quadratic (f_2-f_1) DPOAEs were measured. In addition, in another group of animals the endocochlear potential (EP) was monitored. Both lowering the Ca^{2+} and nimodipine affected the time-varying alterations in the f_2-f_1 in a complex manner: initially decreasing the magnitude and altering the change in response to continuous primaries. BayK application increased the magnitude of the f_2-f_1 and decreased the change in f_2-f_1 in response to continuous primary exposure. Nimodipine decreased the EP and BayK increased the EP. These results are in harmony with the hypothesis that the time-varying amplitude alterations of the f_2-f_1 DPOAE during continuous, primary tone stimulation is sensitive to changes in the function of L-type Ca^{2+} channels and Ca^{2+} levels in the cochlea. In addition,

it appears that the EP is sensitive to alterations to Ca^{2+} channel antagonists and activators. The site of action of the drugs remains to be determined.

Keywords: Cochlear mechanics; Outer hair cells; L-type Ca^{2+} channels; Endocochlear potential, Distortion products, Otoacoustic emissions

Abreviation: T-VAA: time-varying alteration in the amplitude of f_2-f_1 DPOAE by continuous primary stimulation (f_1 = 6.25 kHz, f_2 = 7.5 kHz, L_1 = L2 = 60 dB SPL).

1. Introduction

The quadratic nonlinearity, f_2-f_1, measured as a distrotion product otoacoustic emission (DPOAE), displays time-varying amplitude alterations during continous, primary tone stimulation: During the contiunous primary tone stimulation, after a period of silence, the f_2-f_1 magnitude undergoes a short period of amplitude growth followed by a large reduction in amplitude (Brown, 1988; Kirk and Johnstone, 1993; Kujawa et al., 1994; Kujawa et al., 1995; Whitehead et al., 1991; Lowe and Robertson, 1995). In addition, Kujawa et al., (1995) recently described that if the period of reduction is followed a 1 min period of silence the amplitude undergoes an additional supression yielding what we have called an 'off-effect' which rapidly increases in magnitude. We have elected to abreviate this phenomina of time varying amplitude alteration in f_2-f_1 during continuous primary tone stimulation "T-VAA" in the present paper. Similar amplitude alterations have not been observed in the cubic nonlinearity corresponding to the frequency $2f_1$-f_2 (Kujawa et al., 1995).

The cellular and molecular mechanisms underlying DPOAEs and their alterations over-time remains unclear. Investigations demonstrate that the MOC efferent neurotransmitter, acetylcholine (ACh), acts on the OHCs to suppress DPOAEs (Kujawa et al, 1992?; Kujawa et al., 1993; Kujawa et al., 1994). In addition, there is sufficient evidence to suggest that there is some degree of tonic

influence of ACh on the magnitude of the measured DPOAEs, since antagonists of ACh invaryable increase the magnitude of the DPOAEs (Kujawa et al., 1993, 1994, 1995). Although, sectioning of the efferents has little effect (Littman et al.; Kujawa et al., 1995). Thus ACh may have a small influence on the T-VAA response (Kujawa et al., 1995). In contrast to suggestions to the contrary (Kirk and Johnstone, 1993), there is sufficient evidence to show that ACh and the MOC efferents do not account for these alterations in the f_2-f_1 DPOAE (Kujawa et al., 1995 and Lowe and Roberston, 1995).

Previously, we had demonstrated that lowering the Ca^{2+} levels in perilymph induced a suppression of the cochlear electrical potentials, especially the distortion product known as the summating potential (SP; Bobbin et al., 1991). In addition, nimodipine, a L-type Ca^{2+} channel antagonist, reduced the magnitude and reversed the polarity of the SP (Bobbin et al., 1990). At the time, we speculated that the actions of the treatments were due to alterations in the function of Ca^{2+} and L-type Ca^{2+} channels in the OHCs (Bobbin et al., 1990, 1991). L-type Ca2+ channels are present in OHCs of chick and guinea pig (Fuchs et al., 1990; Chen et al., 1995; Nakagawa et al., 1991; Nakagawa et al., 1992). Isolated OHC function is altered by changing Ca^{2+} levels inside and outside the cell (Pou et al., 199?; Dulon et al.,). DPOAEs are a reflection of the distortion products observed in the motion of the cochlear partition (Robles et al., 1991). In turn, the motion of the cochlear partition is thought to reflect the active motion of the

OHCs at low sound intensities (Robles et al., 1991; Ruggero and Rich, 1991; Mammano and Ashmore, 1993). Thus it is reasonable to predict that DPOAES will be altered by changes in Ca^{2+} levels in the perilymph and by drugs that act on Ca^{2+} channels.

Therefore, one purpose of the present investigation was to test whether changes in Ca^{2+} perilymph levels influenced the magnitude of the T-VAA response. In addition, we tested the hypothesis that the L-type Ca^{2+} channels on OHCs is involved in cochlear mechanics by testing the effects of nimodipine, an L-type Ca2+ channel antagonist, and BayK, an L-type Ca2+ channel activator, on the T-VAA response. Preliminary results have been presented previously (Kujawa et al., 1995).

2. Method

2.1. Subjects

Experiments were performed on pigmented guinea pigs of either sex weighing between 250 and 400 g. Anesthetized animals (urethane, Sigma; 1.5 g/kg, i.p.) were tracheotomized and were allowed to breath unassisted. ECG and rectal temperature were monitored throughout each experiment and temperature was maintained at 38±1°C by a heating pad. Additional urethane was administered as required to maintain an adequate depth of anesthesia.

Surgical procedures have been described previously (Kujawa, et al.,1992a, 1992b). Briefly, cartilaginous ear canals were exposed and partially removed to allow optimum coupling to the sound delivery system. In all animals the right auditory bulla was exposed using a ventrolateral approach and tendons of the right middle ear muscles were sectioned.

2.2. DPOAE: Stimulus generation and response monitoring

The instrumentation employed in these experiments has been described (Kujawa et al., 1994) except that some experiments were carried out utilizing equipment made by Tucker Davis Inc (Gainsville, FLA). Briefly, quadratic (f_2-f_1 = 1.25 kHz) and cubic

($2f_1$-f_2 = 5 kHz) DPOAEs were elicited by equilevel primary stimuli (f_1 = 6.25kHz; f_2 = 7.5 kHz) generated by oscillators, routed through attenuators to separate speakers, and delivered to the right ear of each animal by an acoustic probe assembly. Output from the probe microphone was led via a microphone preamplifier to a dynamic signal analyzer for for Fast Fourier Transform (FFT) analysis (averaging 10 discrete spectra) and spectral display (span = 1 kHz; CF = DP frequency; BW = 3.75 Hz). The noise floors associated with these display windows averaged approximately -15 dB SPL for the f_2-f_1 DPOAE and -18 dB SPL for the $2f_1$-f_2 DPOAE when measured at points ±50 Hz from the DP frequency.

As shown in Fig. 1, continuous, moderate-level (60 dB SPL) primary stimulation generated the same response pattern on f_2-f_1 DPOAE at 1.25 kHz as described by us previously and abbreviated herein as T-VAA for the "time-varying alteration in the amplitude of f_2-f_1 DPOAE by continuous primary stimulation (f_1 = 6.25 kHz, f_2 = 7.5 kHz, L_1 = L2 = 60 dB SPL)" (Kujawa et al., 1995). The following stimulation and response monitoring protocol was employed to generate the T-VAA: 100 consecutiive 10-spectra averages of distortion product amplituded were obtained during continuous primary stimulation. Each of these averages required approximately 5s to complete for a total of 500 s (8.3 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 10-spectra averages

of distortion product amplitude wre obtained (total time approximately 200 s or 2.3 min of stimulation). Six points (A-F) were identified on each response amplitude function of the T-VAA (see Fig. 1). These values were used to characterize the response as previously described (Kujawa et al., 1995). They include an "on-effect" (A-B), a "slow decline" (B-C), on "off-effect" (C-D) and a "second on-effect" (D-E). Here and for all subsequent stimulus manipulations, a 15 min period of rest from primary stimulation separated each test condition from the next.

2.3. Endocochlear potential (EP) experiments

The methods used to measure the endocochlear potential (EP) were similar to those previously described (Bobbin et al., 1990). Briefly, the bone over the basal turn scala media was shaved and a small hole was made through the thinned bone. A glass microelectrode, filled with 150 mM KCl and connected to a DC amplifier (Grass P15) was passed through the hole and inserted into the scala media to record the EP. The output of the amplifier was connected to a digital voltmeter and chart recorder to obtain hard copy records of the EP values.

2.4. Cochlear perfusion experiments

Perfusion studies were undertaken using methods described previously (Kujawa, et al., 1992a; 1992b). The artificial perilymph had a composition of (in mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; NaH_2PO_4, 1; $MgCl_2$, 1; glucose, 11; $NaHCO_3$, 12. The $CaCl_2$ and the NaH_2PO_4 were omitted to obtain the zero Ca^{2+} artificial perilymph perfused and also used to dissolve and test the 2 mM EGTA, the 5 mM BAPTA and the 2 and 4 mM Mg^{2+}. Nimodipine (Research Biochemicals International) and BayK [S(-)-Bay K 8644, Research Biochemicals International] were dissolved in dimethylsulfoxide (DMSO, Sigma) and stored in the dark at -20°C for no more than one week. The nimodipine in DMSO, BayK in DMSO, and DMSO alone were mixed with the artificial perilymph on the day of use at desired concentrations. Otherwise, all perfusion solutions were freshly prepared on the day of use. The pH of all solutions was adjusted to 7.4 when necessary. Perfusates were introduced into the cochlear perilymph at room temperature and at a rate of 2.5 μl/min for 10 or 15 min through a hole in basal turn scala tympani and were allowed to flow from the cochlea through an effluent hole placed in basal turn scala vestibuli. Effluent was absorbed within the bulla using small cotton wicks. In all animals, the first two perfusions were of artificial perilymph alone. These perfusions were accomplished to achieve a stable baseline to which subsequent alterations in the artificial perilymph and drug-related changes could be compared. These perfusions were followed by perfusions of the altered artificial perilymph or experimental drug.

Effects of treatments were quantified using repeated measures analysis of variance (ANOVA) and Newman-Kuels multiple range test. The care and use of the animals reported on in this study were approved by LSUMC's Institutional Animal Care and Use Committee.

3. Results

3.1. Actions of low Ca^{2+}, high Mg^{2+}, EGTA and BAPTA on DPOAE

The cochlear perfusions in the following experiments describing the effects of perfusions on the T-VAA were carried out for 10 min. This was followed by a 15 min period of silence where no primaries or other sounds were delivered to the ear of the animal. Then the f_2 and f_1 primaries were delivered to obtain the f_2-f_1 DPOAE in the T-VAA response. Data were obtained from five animals and are shown in Fig. 2.

Perfusion of the cochlea with artificial perilymph solution without added Ca^{2+} had very little effect on the overall shape of the T-VAA (Fig. 2). The most obvious effect was a small (about 2 dB) reduction in the starting value (point A in Fig. 1). Subsequent perfusions of solutions containing no added Ca^{2+} with added Mg^{2+} (2 mM and 4 mM) resulted in greater suppression of the starting value (point A; Fig. 2). The 4 mM Mg^{2+} solution resulted in the reversal of the "off-effect" and the "second on-effect" (points C-D and D-E). All actions were readily reversed with washing with normal artifical perilymph solution (Fig. 2).

BAPTA (5 mM), which is a more powerful agent at complexing Ca^{2+}, when added to the zero Ca^{2+} artificial perilymph appeared to

reverse the whole T-VAA response (Fig. 2). The actions of the BAPTA were reversed with washing with control artificial perilymph solution (Fig. 2).

3.2. Actions of DMSO, nimodipine and BayK on DPOAE

All of the cochlear perfusions in the following experiments were carried out for 15 min. This time period was taken as the 15 min period of silence where no primaries or other sounds were delivered to the ear of the animal. Immediately following the termination of the perfusion, the f_2 and f_1 primaries were delivered at equilevel (60 dB SPL) to obtain the T-VAA response. After this recording (approximately 2 min), the DPOAE amplitude growth functions were recorded first for f_2-f_1 DPOAE and then $2f_1$-f_2 DPOAE with equilevel primaries starting at 70 dB and decreasing in 5 dB steps to 25 dB. Data were obtained from five animals per treatment (nimodipine, 5 animals; DMSO, 5 animals; BayK, 5 animals).

Perfusion of increasing concentrations of DMSO which were the same as those concentrations in the nimodipine and BayK solutions had little effect on the T-VAA response (Fig. 3). The DMSO decrease of the onset value (A) was not significant (Figs. 3 and 4). The DMSO appeared to increase the slope of the "slow decline"

(B-C). In addition, after the perfusion with the 0.01% DMSO solution the "off-effect" or "second on-effect" reversed and after the last perfusion (0.1%) the "off-effect" or "second on-effect" segment had virtually flattened out.

In contrast to the effects of solvent alone, the drugs had large effects. The actions of nimodipine were very complex (Figs. 3 and 4). The starting value (A) was decreased by 0.1 μM - 1.0 μM nimodipine and then the value reversed and began to return towards normal with perfusions of higher concentrations of nimodipine (3 and 10 μM; Figs. 3 and 4). The "on-effect" (A-B) was increased by the same low concentrations (0.1 - 1 μM) whereas the higher concentrations appeared to reverse the "on-effect" (3-10 μM). However, the reversal of the "on-effect" may have been due to the slowly developing reversal of the "slow decline" (B-C) which started with a concentration of 0.1 μM nimodipine and can be seen as a increase in f_2-f_1 magnitude at about 6 min into the recording. With increasing concentrations of nimodipine this increase [or reversal of the slow decline] appears to move earlier in time: 0.1 μM = 6 min; 0.3 μM = 4 min; 1.0 μM = 3 min; and then at 3 and 10 μM it may even start at 0 min. The "off-effect" and the "second on-effect" reversed at the lowest concentration of nimodipine tested (0.1 μM) and remained reversed over the course of the subsequent perfusions with increasing concentrations.

Compared to nimodipine, BayK appeared to be very straight forward in its actions on the T-VAA response (Figs. 3 and 4). BayK increased the overall values obtained for f_1-f_2. BayK increased the onset value (A) and this increase was significant at the 1 - 10 μM concentrations (Fig. 4). BayK did not appear to have much of an effect on the "on-effect" (A-B), the "off-effect" (C-D) or the "second on-effect" (D-E). On the other hand, BayK did decrease the slope of the "slow-decline" (B-C). Interestingly, the overall shape of the T-VAA response after 10 μM nimodipine appears to be the reverse of the T-VAA response after 10 μM BayK (Fig. 3).

Distortion product amplitude growth functions were recorded first for f_2-f_1 and then $2f_1$-f_2 immediately after each perfusion. Results on the growth functions with f_2-f_1 appear to parallel, to some extent, the effects observe on the T-VAA response of f_1-f_2 (Figs. 5 and 6). DMSO had very little effect on the growth function, with the exception that the DMSO appeared to suppress the "knee" in the function which occured at 60 dB SPL (Fig. 5). Nimodipine had complex effects in that low concentrations (0.1 - 0.3 μM) had little effect, a slightly higher concentration (1 μM) enhanced the function, and higher concentrations (3 - 10 μM) suppressed the low intensity portions while enhancing the high intensity portions further (Figs. 5 and 6). BayK enhanced the growth function at all intensities especially at 3 and 10 μM (Figs. 5 and 6). By comparison, neither DMSO or BayK had any effect on

$2f_1$-f_2 growth functions while nimodipine suppresed them, especially in the low intensity range (Fig. 5 and 6).

3.3. *Actions on the endocochlear potential (EP)*

All perfusions in the EP study were 15 min in duration. The perfusion pump was turned on, then the pipette was inserted into the infusion hole and approximately 10 s later the first EP value was recorded as shown in Fig 7 (as zero min values). One min values were then obtained for 15 min after which the pipette was removed and filled with the next solution. Approximately 4 min elapsed between perfusions. The 16 values (0 - 15 min) were then averaged accross animals to obtain the mean values displayed in Fig. 7.

Insertion of the perfusion pipette to start the perfusion resulted in an immediate decrease in the EP that slowly recovered as the perfusion continued (Fig. 7). We assume this is an effect of the increase in pressure and it appeared not to affect the test-retest of the control DMSO perfusions. The first perfusion of artificial perilymph increased the EP. The second perfusion of the artificial perilymph (AP2) did not alter the EP, indicating stability of the EP amplitude. Therefore, as with the DPOAEs, the AP2 value is considered the control for each series of perfusions. DMSO perfusions were controls for the concentration of DMSO in the drug solutions.

Perfusions of increasing concentrations of the drug solvent, DMSO, had no significant effect on the EP when compared to its AP2 (Figs. 7 and 8). The experimental drug nimodipine produced a suppression of the EP that was significantly different ($p < 0.05$) from its paired or equivalent DMSO concentration at all drug concentrations (0.1 - 10 μM). The nimodipine data in Fig. 8 was fitted to the logistic equation using Origin software and indicated that the ED_{50} was 0.42 μM. BayK induced a very slight increase in EP that was significantly different at 10 μM from its paired or equivalent DMSO concentration ($p < 0.05$; Fig. 8).

4. Discussion

4.1. General Alterations in DPOAEs by the treatments

The present work demonstrates that the f_2-f_1 DPOAE, the $2f_1$-f_2 DPOAE and the T-VAA response as initally reported by Kujawa et al., (1995) are all altered by changes in perilymph Ca^{2+} levels and by nimodipine and BayK which are drugs that act on L-type Ca^{2+} channels.

The magnitude of f_2-f_1 DPOAE was suppressed by lowering the Ca2+ levels. Nimodipine, the L-type Ca2+ channel antagonist, also suppressed the magnitude of the f2-f1 DPOAE at low concentrations of the drug. BayK, the L-type Ca2+ channel activator, augmented the magnitude of the f2-f1 DPOAE. These actions of lowering the Ca2+ levels, nimodipine and BayK are consistent with the hypothesis that these three treatments were all acting via L-type Ca2+ channels to affect the f2-f1 DPOAE. The 2f1-f2 DPOAE was unaffected by BayK, affected by nimodipine only at high concentrations of nimodipine, and was only slightly affected by changes in perilymph Ca2+ levels suggesting that this DPOAE is less sensitive to changes alterations in L-type Ca2+ channel activity. In contrast to these straight forward effects, the treatment-induced changes in the T-VAA response were complex. Overall, the results are consistent with the hypothesis that L-type Ca^{2+} channels

are involved in cochlear function. In particular, the channels appear to be necessary for mechanical functioning of the cochlear partition. The site of action of these treatments is speculative at present.

4.2. Sites and mechanisms underlying response to treatments

Several mechanisms might be proposed to account for the changes observed in the DPOAEs by the various treatments. Some of these have been addressed previously (e.g., Bobbin et al., 1990 and 1991) and include a) an L-type Ca2+ channel on OHCs, b) altered Ca2+ levels in endolymph in the scala media, c) a non-specific effect on another ion channel, and d) an L-type Ca2+ channel in the stria vascularis.

L-type Ca2+ channel on OHCs. An L-type Ca^{2+} channel has been described electrophysiologically on OHCs of chick and guinea pig (Fuchs et al., 1990; Chen et al., 1995; Nakagawa et al., 1991; Nakagawa et al., 1992). Previously, we reported that changing Ca^{2+} levels in perilymph and perfusion of the L-type Ca^{2+} channel antagonist, nimodipine, through the perilymph compartment changed in the magnitude and polarity of the SP (Bobbin et al., 1990; Bobbin et al, 1991). We speculated that the changes in SP were due to an alteration in the function of L-type Ca^{2+} channels on OHCs. Likewise the results obtained in the present study may be due to an

action on the same channels. It is well known that the OHCs undergo length changes in response to low levels of stimulation (Ashmore, 1987; Brownell et al, 1985; Dallos et al., 1991; Santos-Sacchi and Dilger, 1988; Mammano and Ashmore, 1993). This length change is responsible for the sharp tuning of the cochlear partition (Ruggero and Rich, 1991; Mammano and Ashmore, 1993) and the presence of DPOAEs in the movement of the cochlear partition (Robles et al., 1991). The role of the L-type Ca^{2+} channels in the OHC length change, if any, is not known. The changes in OHC length are related to the low level sound-evoked DPOAEs in a complex fashion. For example, changes in the function of the OHCs induced by activation of the MOC efferents change the magnitude of the DPOAEs, especially at low levels of sound (Kirk and Johnstone, 1993; Kujawa et al.1993; Kujawa et al., 1994; Kujawa et al., 1995; Puel and Ribillard, 1988). Thus it seems reasonable to speculate that BayK augmented the activity of the L-type Ca^{2+} channels on the OHCs and this augmented the DPOAEs, whereas nimodipine decreased the activity of the L-type Ca^{2+} channels and this depressed the DPOAEs.

One of the problems with the hypothesis that the L-type Ca^{2+} channels on OHCs explain our results is the level of depolariztion required to activate the channels. Most reports of in vivo recordings show that the sound-evoked depolarization obtained in the OHCs is usually less than 10 mV (e.g., Dallos and Cheatham, 1992). Yet for L-type Ca^{2+} channels to be activated the membrane potential would have to depolarize 30 mV to bring the membrane

potential to -40 mV (Chen et al., 1995). Recordings from OHCs have indicated that their normal resting membrane potential is around -70 mV in vivo (e.g., Dallos and Cheatham, 1992). Thus most evoked responses reported from OHCs are below the magnitude necessary to reach the threshold of activation of the L-type Ca^{2+} channels. Although it has been reported that both OHCs and IHCs can produce peak-to-peak responses as large as 40 mV (Dallos and Cheatham, 1992). In additon as pointed out by Dallos and Cheatham (1992), the small depolarization response usually obtained from OHCs in response to sound may be a reflection of the difficulty in obtaining good quality recordings from OHCs. Finally, the activation curve reported for the L-Ca^{2+} channels in vitro may be different in vivo. Therefore, further research is necessary to prove that altered L-type Ca^{2+} channel activity in OHCs caused the results obtained in the present study.

Ca2+ levels in endolymph in the scala media. An additional mechanism that could account for the results obtained is an alteration in the Ca^{2+} concentration in the scala media fluid compartment or the endolymph. Tanaka and Salt (1994) reported that cochlear function as measured via EP, SP and CAP was highly sensitive to small disturbances in Ca^{2+} concentration in endolymph. Others have shown effects of Ca^{2+} on adaptation and transduction at the level of the steriocillia (Hudspeth, 1983; Assad et al., 1991). So lowering the concentration of Ca^{2+} in perilymph may have altered the concentration of Ca^{2+} at the steriocillia and altered their

function. However, nimodipine and BayK should not affect overall Ca^{2+} levels in the endolymph or in other tissues, since the ion levels are controlled by transport and diffusion. Instead, the drugs must be acting on a protein, for example L-type Ca^{2+} channels.

Non-selective effect on another ion channel. The drugs used in this study are known to affect ion channels other than L-type Ca^{2+} channels. For example, Lin et al., (1991) reported that verapamil an L-type Ca^{2+} channel blocker similar to nimodipine will block a late K^{+} current found in OHCs. Therefore, an effect on such ion channels may have produce the effects observed in the present results.

L-type Ca^{2+} channel in the stria vascularis. Another mechanism that may have produce the results observed is a change in the value of the EP, the +90 mV in the scala media. Others have shown that distortion products observed in the motion of the basilar membrane and those recorded in the ear canal (i.e., OAEs) are sensitive to alterations in the magnitude of the EP (Rugerrio et al,???; Mills et al., 1993; Whitehead et al.,???). This sensitivity is related to the fact that the EP is necessary to power the motor moving the OHCs in response to sound (Dallos and Evans, 1995). Mills et al., 1994 and Schmiedt and Adams, 1981, hear res 5, 295. ?? report a suppression or a reversal in the magnitude of f_2-f_1 DPOAE similar

to the present results obtained with lowering of the Ca^{2+} concentration and with nimodipine treatment.

In most reports altering perilymph Ca^{2+} levels does not induce changes in EP (Konishi and Kelsey, 1970; Sato, 1989; Bobbin et al., 1991). Even the addition of Ca^{2+} chelators such as EGTA to the perfusate seems to have little effect (2 mM = Bobbin et al., 1991; 4 mM EGTA = Konishi and Kelsey, 1970; and 4 and 10 mM EGTA = Sato, 1989). There are large potential pools for Ca^{2+} in the many fluid compartments of the cochlea, including blood vessels and cerebral spinal fluid entering via the cochlear aqueduct. Given this then it is not surprising that simple perfusion of low levels of Ca^{2+} through the perilymph compartment would have little impact on the function of the cochlea or on EP magnitude.

On the other hand, nimodipine and BayK are effective at the receptors of L-type Ca^{2+} channels even in the presence of high levels of Ca^{2+} (Chen et al, 1995). Thus they would not be affected by the large pools of Ca^{2+} in the tissue. This may explain why nimodipine significantly reduced EP at 0.1 μM, the lowest concentration tested. Increasing the concentration of nimodipine resulted in a greater reduction in EP with a maximum reduction of 12 ± 1.2 mV apparently being reached at 10 μM. This change is close to the 15 ± 3 mV change in EP induced by a single 30 min perfusion of 10 μM nimodipine reported previously by us (Bobbin et al., 1991). BayK increased EP, an effect which should occur if

BayK is "activating" an L-type Ca2+ channel as it does in OHCs (Chen et al., 1995) and nimodipine is blocking the same L-type Ca^{2+} channel to reduce EP. To our knowledge, no one has reported the presence of an L-type Ca2+ channel in the maginal cells of the stria vascularis, the structure generating the EP (Offner et al., 1987; Sunose et al., 1994). Therefore, the location of the putative L-type Ca^{2+} channel remains to be determined. However if this hypothesis as to the mechanism of action of BayK and nimodipine is correct, then the role of the L-type Ca^{2+} channel in generating the EP is significant, but relatively small (i.e., < 10 %).

Interestingly, the concentration of nimodipine (0.1 μM) we found effective is in the range of the serum concentrations found in patients taking the drug (Raemsch et al., 1985). We studied perilymph levels of the drug and not serum levels, so we do not know the serum levels affecting the EP. But it is intriguing that a clinically used drug may be available to reduce the EP in patients. In this regard it may be used to examine the impact of a small reduction in EP on such disease states as tinnitus and Mineare's as suggested by others (e.g Guth and Norris et al; jasterboff).

We do not know whether these small changes in EP by BayK (+2.3 mV) and nimodipine (-12 mV) are sufficient to account for the changes in DPOAE, in particular the decrease in f_2-f_1 by low doses

of nimodipine and the increase in f_2-f_1 by BayK. Mills et al., (1995) report that the cubic DPOAE is proportional to the square of the EP. In addition, it is possible that the alteration in the function of the L-type Ca^{2+} channel located at a site remote from the stria affected the EP. For instance, blockade of the L-type Ca^{2+} channel in the OHCs by nimodipine may have induced the reduction in EP recorded. We know too little about the function of the channels on the OHCs to even speculate as to how this might come about.

In summary, we have shown previously (Bobbin et al., 1990; Bobbin et al., 1991) that both lowering perilymph Ca^{2+} levels and perilymph application of nimodipine decrease the magnitude, and even reverse the sign, of the distortion product known as the SP. Results in the present study extend this effect to DPOAEs, in particular the f_2-f_1 and the T-VAA. These results support the hypothesis that Ca^{2+} and L-type Ca_{2+} channels are involved in the function of the OHCs, EP production and DPOAEs. However, the site of action of the three treatments utilized in the present study remains to be determined with certainty.

Acknowledgments

The authors wish to thank David Owens for computer programming, and Elaine McDonald and Amy Bayber for technical help. This research was funded in part by NIH research grant DC00722 and training grant DC00722, DAMD 17-93-V-3013, Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation.

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Figure Legends

Fig. 1. A typical example illustrating the T-VAA response: "time-varying alteration in the amplitude of f_2-f_1 DPOAE by continuous primary stimulation (f_1 = 6.25 kHz, f_2 = 7.5 kHz, L_1 = L2 = 60 dB SPL)". Each data point represents a 10 spectra average and required 5 s to complete. Break in response amplitude trace (C-D) represents 1 min with no primary stimulation. Points A-F thus identified in the trace were used to describe changes and to calculate magnitudes of component amplitude changes.

Fig. 2. Effect of altering Ca^{2+} and Mg^{2+} levels on the T-VAA. (A, B and C): Response amplitude (means; N = 5 animals) recorded following successive perfusions in the same animals of: the second control perfusion (AP2), zero Ca^{2+}, zero Ca^{2+} with 2 mM Mg^{2+}, zero Ca^{2+} with 4 mM Mg^{2+}, a wash perfusion (wash 2), zero Ca^{2+} with 5 mM BAPTA, and a final wash (wash 2b). Only every 5th data point is plotted for clairity. Pooled standard errors were: AP2 = 1.20; 0 Ca2+ = 0.75; 2 mM Mg2+ = 1.19; 4 mM Mg2+ = 2.55; Wash 2 = 0.72; 5 mM BAPTA = 1.62 and wash 2b = 1.42. See legend for Fig. 1 for additional information.

Fig. 3. Effect of perfusion of nimodipine (n = 5 animals), DMSO (n = 5 animals) and BayK (n = 5 animals) in increasing concentrations following the control perfusion (AP2) on the T-VAA. Mean response amplitude for every data point is shown. The value for the pooled ± S.E. for all the data within a small frame is shown in the upper right hand corner of that frame. The dashed line is drawn at 15 dB for visual reference. For additional explanation see legend for Fig. 1.

Fig. 4. Effect of perfusion of nimodipine, DMSO and BayK in increasing concentrations following the control perfusion (AP2) on the first value obtained in recording the T-VAA and shown in Fig. 3 (1 - 10 s; mean ± S.E.). The significant difference of several of the nimodipine and BayK data points from both their respective AP2 and DMSO-alone concentration is illustrated; while in the DMSO experiments, none of the DMSO data points were different from the AP2 (* $p < 0.05$; ** $p < 0.01$; N = 5 animals per drug treatment). For additional explanation see legends for Fig. 1 and 3.

Fig. 5. Effect of perfusion of nimodipine, DMSO and BayK in increasing concentrations following the control perfusion (AP2) on the input/output growth functions of f_2-f_1 and $2f_1$-f_2 DPOAEs. The functions were obtained immediately following the T-VAA (f_2-f_1 followed by $2f_1$-f_2). The mean response amplitudes are shown for only those concentrations which had some effect (N = 5 animals per drug treatment). The symbols for the concentrations are the same for both f_2-f_1 and $2f_1$-f_2. Dotted line approximates the noise floor. For representative S.E. and statistics see Fig. 6.

Fig. 6. Effect of perfusion of nimodipine, DMSO and BayK in increasing concentrations following the control perfusion (AP2) on the DPOAE amplitude obtained in response to primary levels of 55 dB SPL. The values are taken from those shown in Fig. 5 which were recorded immediately following the T-VAA (mean ± S.E.). The significant difference of several of the nimodipine and BayK data points from both their respective AP2 and DMSO-alone concentration is illustrated; while in the DMSO experiments, none of the DMSO data points were different from AP2 (* $p < 0.05$; ** $p < 0.01$; N = 5 animals per drug treatment).

Fig. 7. Effect of perfusion of nimodipine, DMSO and BayK in increasing concentrations following the control perfusion (AP2) on the EP (n = 5 animals per drug). Shown are the values (mean + S.E.) obtained immediately (within 10 s) after the insertion of the perfusion pipette (zero min value) followed by the 15 one min values following the zero min value. Approxiamately 4 min elapsed between perfusions and data collection. Average drift was nimodipine records = mV; DMSO records = mV; and BayK = mV.

Fig. 8. Dose response curve of the effect of nimodipine, DMSO and BayK on the EP. For each experiment, the last 15 min value obtained during perfusion of the second control perfusion (AP2) was subtracted from the last (15 min) EP value during perfusion of each concentration of drug shown in Fig. 7. The mean ± S.E. of each value calculated in this manner is shown (N = 5 animals per drug treatment). The significant difference of several of the nimodipine and BayK data points from their respective DMSO-alone values is illustrated (* $p < 0.05$; ** $p < 0.01$).

Fig 1

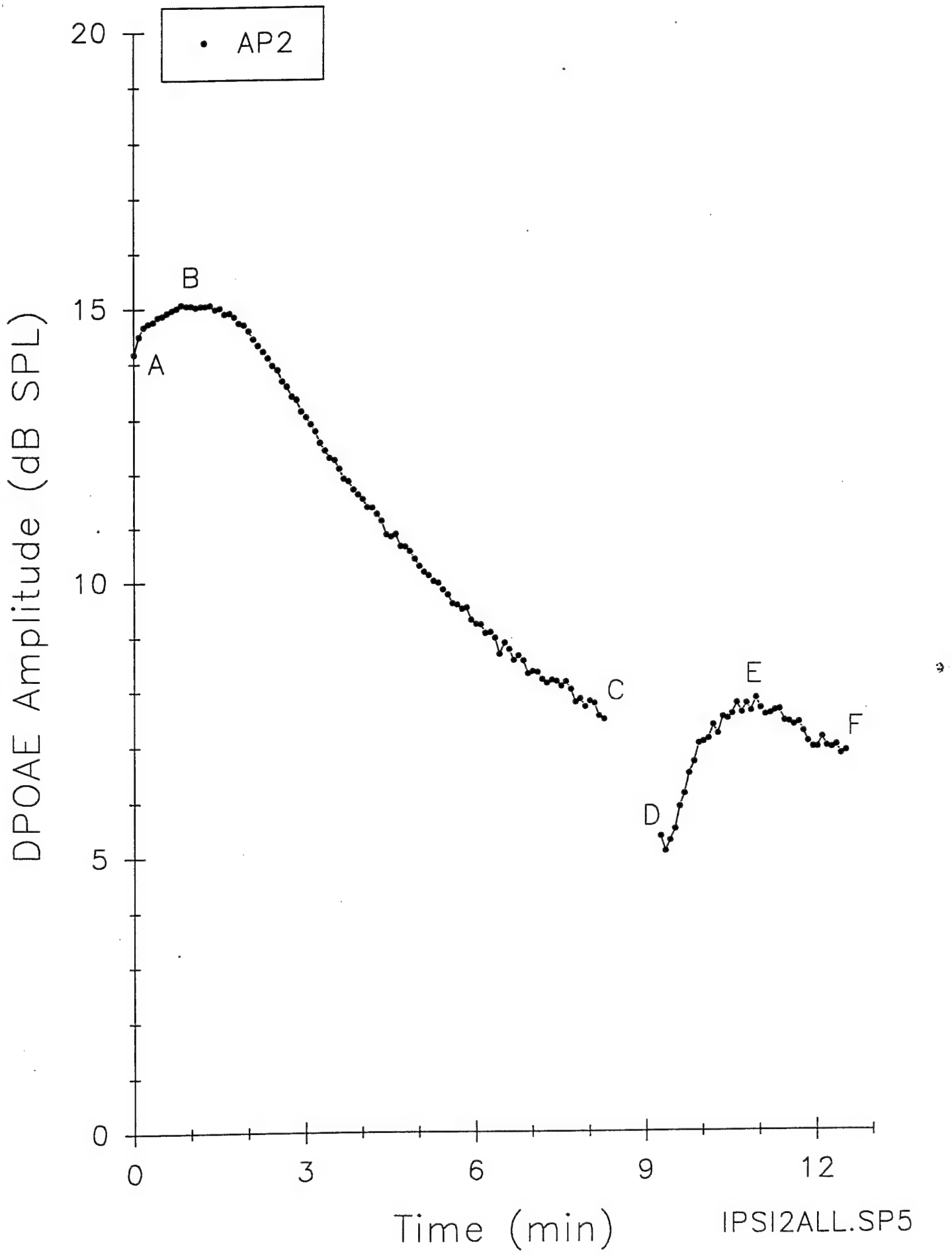

AP2
20
15
10
5
0
DPOAE Amplitude (dB SPL)
A
B
C
D
E
F
0
3
6
9
12
Time (min)
IPSI2ALL.SP5

Fig 2

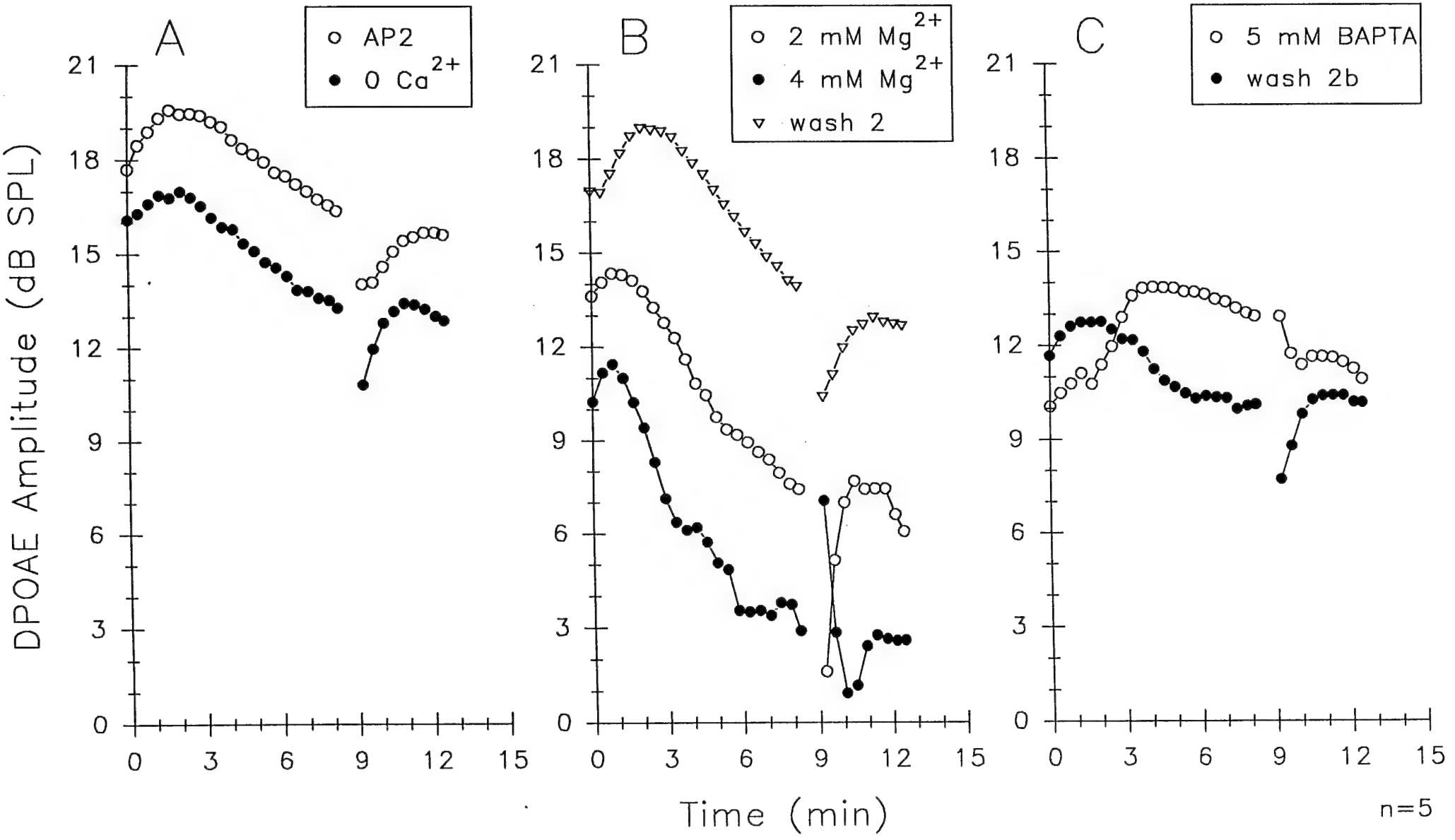

A
AP2
0 Ca2+
B
2 mM Mg2+
4 mM Mg2+
wash 2
C
5 mM BAPTA
wash 2b
DPOAE Amplitude (dB SPL)
Time (min)
n=5
Ca_BAPTA.SP5

Fig 3

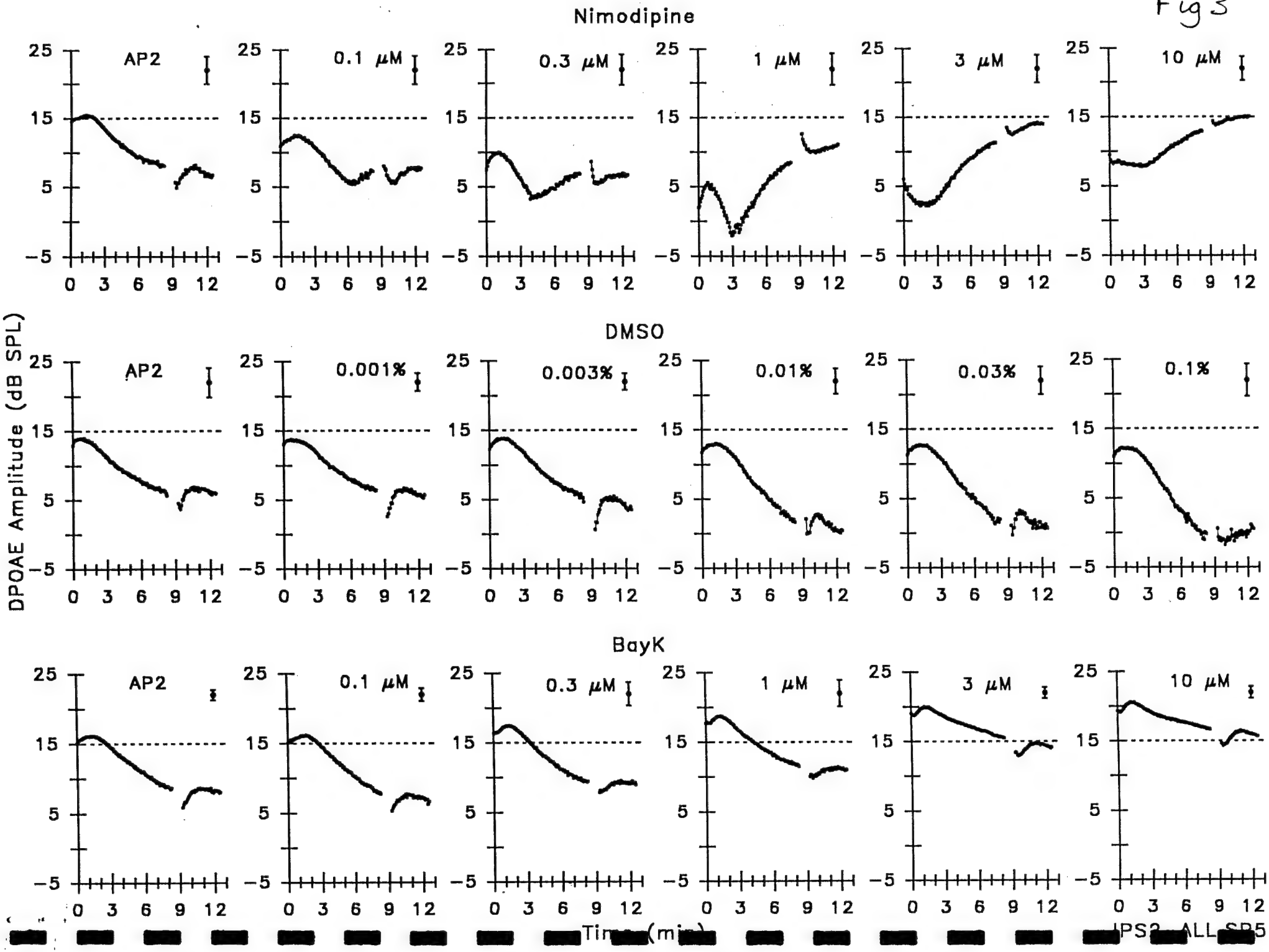
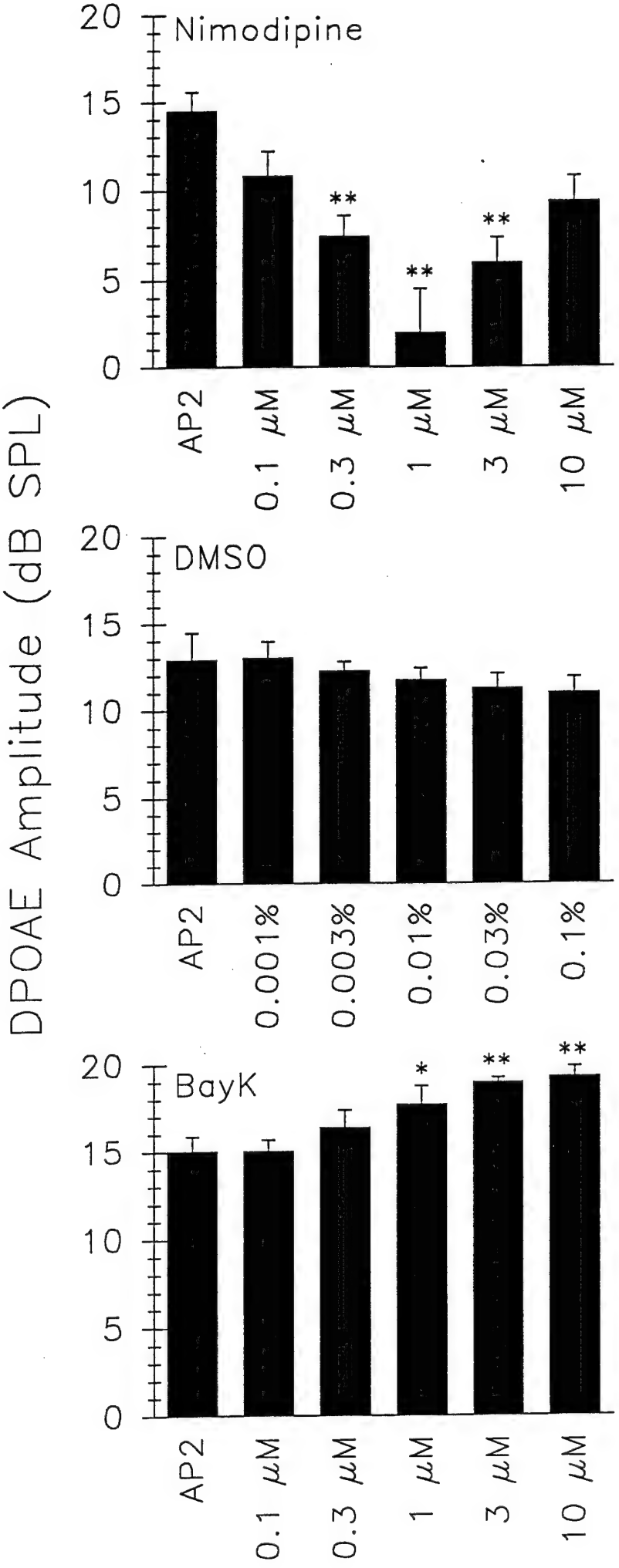
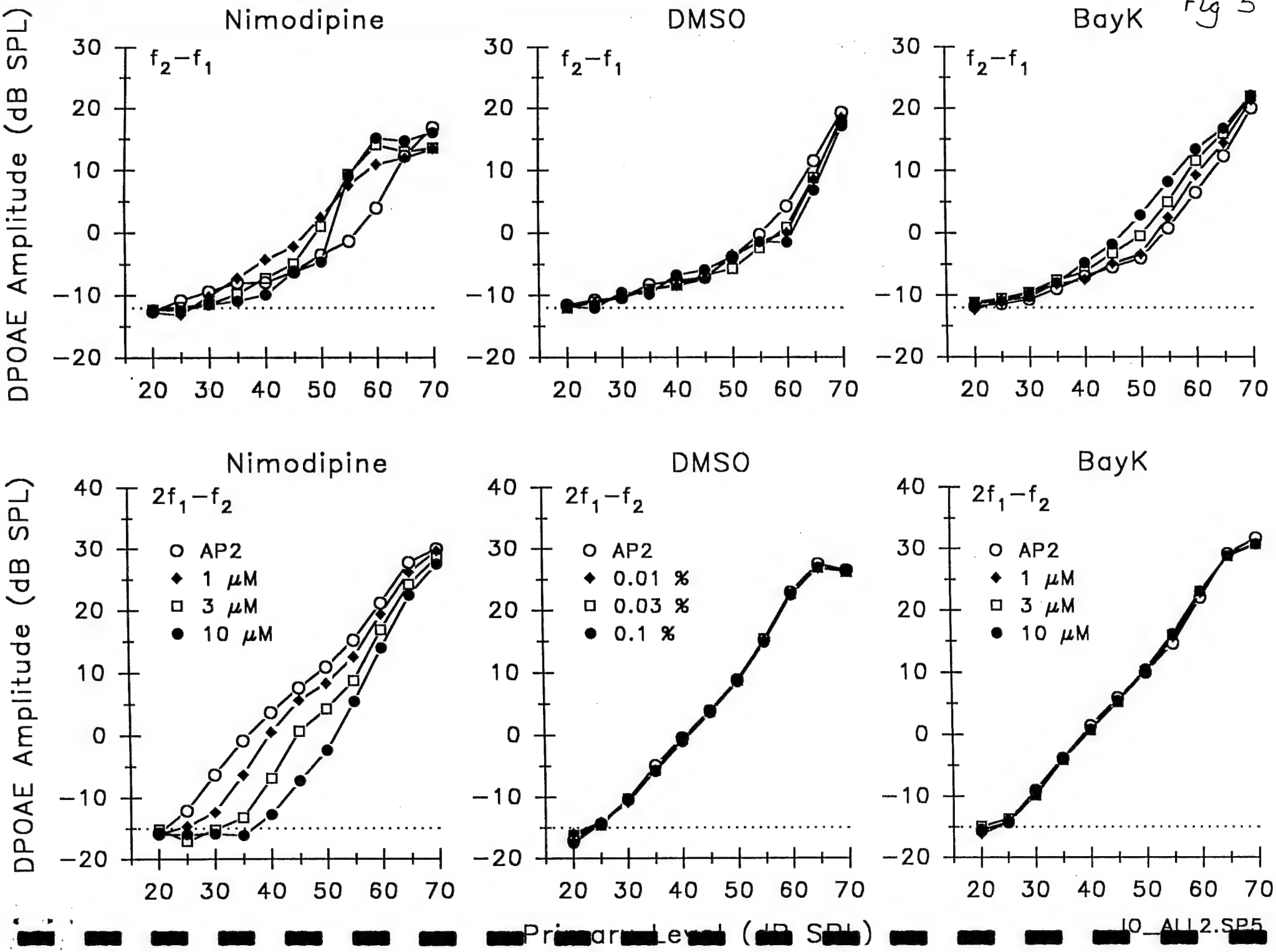

Nimodipine
AP2
0.1 μM
0.3 μM
1 μM
3 μM
10 μM
DMSO
AP2
0.001%
0.003%
0.01%
0.03%
0.1%
BayK
AP2
0.1 μM
0.3 μM
1 μM
3 μM
10 μM
DPOAE Amplitude (dB SPL)
25
15
5
-5
0 3 6 9 12
Time (min)
IPS2 ALL SP5

Fig 4



A-F_IP.SP5

Fig 5


Nimodipine
DMSO
BayK
f_2-f_1
$2f_1-f_2$
DPOAE Amplitude (dB SPL)
AP2
1 μM
3 μM
10 μM
0.01 %
0.03 %
0.1 %
Primary Level (dB SPL)
IO_ALL2.SP5

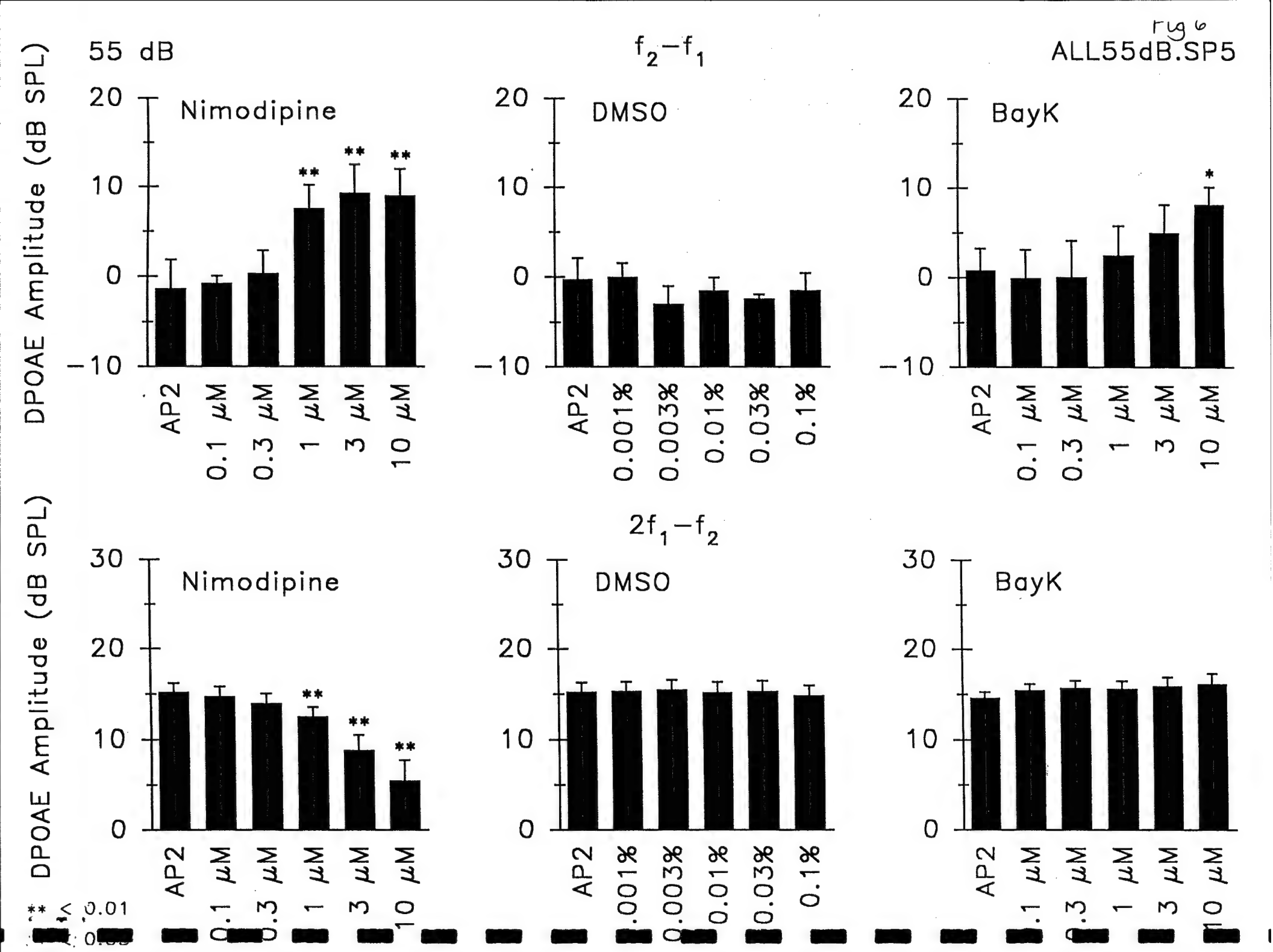


Fig 6
ALL55dB.SP5
55 dB
f_2-f_1
$2f_1-f_2$
DPOAE Amplitude (dB SPL)
Nimodipine
DMSO
BayK
AP2
0.1 μM
0.3 μM
1 μM
3 μM
10 μM
0.001%
0.003%
0.01%
0.03%
0.1%
**
*

1. Introduction

Certain levels of continuous noise exposure result in a decrease in auditory sensitivity. This change in sensitivity becomes larger during the first 24 hours of exposure and then plateaus as the exposure continues. This pattern of sensitivity change is referred to in the behavioral literature as an asymptotic threshold shift (ATS). ATS has been demonstrated in chinchillas (Carder and Miller, 1972; Mills, 1973, 1976; Bohne and Clark, 1982; Clark and Bohne, 1987), monkeys (Moody et al., 1976), guinea pigs (Syka and Popelar, 1980), and humans (Melnick, 1976)]. ATS remains stable during exposure lasting as long as three years (Clark and Bohne, 1987).

The minimum level of noise exposure required to produce ATS depends upon the band of noise used and the species of animal under investigation. In chinchillas, Carder and Miller (1972) suggested that a 0.5 kHz octave band noise should not induce an elevation of auditory threshold when the exposure level is at or below 65 dB SPL. This was supported by Bohne (1976) who observed no structural damage to chinchilla hair cells after 9 days of exposure to a noise of 65 dB SPL and the same spectral characteristics. On the other hand for an octave band of noise centered at 4.0 kHz, Mills (1973) suggested that a level at or below 47 dB SPL would not produce ATS in chinchillas. According to these results, as the center frequency of the noise band increases, the exposure level required to produce ATS decreases.

To date there is no information about the level of noise required to produce ATS in guinea pigs. In guinea pigs, Syka and Popelar (1980) demonstrated the existence of an ATS over the course of a 5 day exposure to a 100 dB SPL third octave band of noise centered at 2 kHz. However, the authors did not explore the minimum level of noise necessary. The level was much more than the minimum because a significant threshold shift was still present 120 days post-exposure.

Whether ATS is due to changes in cochlear structure or function is unclear. Although the level of ATS remains stable for exposure durations lasting as long as three years (Clark and Bohne, 1987), anatomical evidence suggests that with increased exposure duration, there is an increase in cochlear damage (Bohne and Clark, 1982). Canlon and Fransson (1995) exposed guinea pigs to a 1 kHz pure tone at 81 dB SPL for 24 days. They did not perform behavioral measures to determine if an ATS existed. However, they did report that this exposure did not cause any significant changes in auditory brainstem response thresholds and DPOAE amplitudes of the noise-exposed animals as compared to the unexposed animals. In addition, analysis of surface preparations of the organ of Corti at the light microscopic level did not reveal any significant hair cell loss after the long-term exposure.

In our laboratory, Chen et al. (1995) recently reported that an 11 day exposure to a 65 dB SPL narrowband noise (1100 - 2000 Hz) was sufficient to produce changes in the response of isolated OHCs to ATP application. This level of noise is close to the minimum levels suggested to induce changes that would be observed as ATS in behavioral tests of chinchillas (Carder and Miller, 1972; Mills, 1973). In addition, it suggests that this level of noise may induce changes in cochlear function. In general, it is difficult to measure auditory function behaviorally in the guinea pig. Therefore, to test the hypothesis that the noise used by Chen et al., alters cochlear function, we examined the effects of the chronic low-level noise exposure on DPOAEs. DPOAEs measurements were chosen for study since they are believed to reflect the status of OHC function (Mountain, 1980; Siegel and Kim, 1982a,b). In addition, we examined other measures of OHC function and cochlear mechanics by investigating whether the time-varying amplitude characteristics of the quadratic DPOAE and the amount of contralateral suppression of the DPOAEs are altered.

2. Methods

2.1. Subjects

A total of 39 pigmented guinea pigs of either sex (300-500 g) was used as subjects. Upon delivery from the supplier, animals from the same litter (or at least from the same delivery date) were randomly assigned to three groups (n=13/group) according to the number of days exposed to a low-level continuous noise (0, 3, and 11 days). The control group was not exposed to the noise. These animals were housed at the university's animal care facility. The experimental groups were exposed to a 65 dB SPL (A-scale) narrow band noise (NBN, cutoff frequencies 1.1 and 2.0 kHz) 24 hours/day for 3 and 11 days, respectively. These animals were tested immediately upon removal from the noise. Only animals with a normal Preyer reflex and a normal otoscopic examination were included in the study. All animals were given free access to food and water. The care and use of the animals that were used in this study were approved by LSUMC's Institutional Animal Care and Use Committee.

2.2. Noise exposure facility and noise generation

Unanesthetized guinea pigs were housed in groups of 10 or less in a small sound-attenuated booth (approx. interior dimensions 76 × 60 × 40 cm; Industrial Acoustics Company, Inc.) contained within a larger sound-attenuated booth (Industrial Acoustics Company, Inc.). The walls of the smaller booth were lined with carpet. The speaker was mounted on a wooden surface which was positioned approximately 40 cm above the level of the guinea pigs' ears. The floor of the booth was lined with a metal

pan filled with animal bedding (Sani-Chips). A small light was installed within the booth and controlled with a timer to provide the animals 12 hours of light and 12 hours of darkness.

The noise was generated by a WG2 Waveform Generator (Tucker-Davis Technologies) which was set to produce a uniform noise signal. This signal was filtered using a Brickwall Filter (Wavetek/Rockland Model 753A) configured in the bandpass mode with a low frequency cutoff at 1.1 kHz and a high frequency cutoff at 2.0 kHz. The level of the noise was controlled by a PA4 Programmable Attenuator (Tucker-Davis Technologies). Additional power was gained using a power amplifier (McIntosh MC2100) producing the final signal delivered to the speaker (Realistic 40-1286C; 8Ω, 30 watts).

Noise levels were monitored by placing a sound level meter (Brüel & Kjaer Type 2230 Precision Integrating Sound Level Meter) within the booth. The microphone was positioned so that it was approximately at the level of the guinea pigs' ears and was placed at various positions around the booth to ensure that the noise was equally distributed throughout the booth. The spectrum of the noise as measured with a signal analyzer (Hewlett-Packard 3561A) is shown in Fig. 1. The voltage across the speaker (approx. 46-48 mV) was also checked on a daily basis using a digital voltmeter (Wavetek Corporation BI-DM15XL).

2.3. General surgical methods

Immediately prior to testing, the animals were anesthetized by administering a dose of urethane (Sigma; 1.5 g/kg, i.p.), tracheotomized, and allowed to breathe unassisted. Supplementary doses of anesthetic (urethane, 1.5 g/kg) were given to maintain an adequate depth of anesthesia (as indicated by a lack of a withdrawal response to deep pressure and pain applied to the animal's paw). ECG and rectal temperature were monitored throughout each experiment and temperature maintained at 38 ± 1°C using a heating pad.

The surgical procedures used were similar to those described by Kujawa et al. (1992, 1993). Briefly, cartilaginous ear canals were exposed and partially removed to allow for proper placement of hollow ear bars which help secure the animal in the headholder. This procedure also ensured optimal coupling of the sound delivery system to both ears. Using a ventrolateral approach, the ipsilateral (right) auditory bulla was exposed and opened to gain access to the tendons of the middle ear muscles. These tendons were sectioned in all animals to prevent the involvement of middle ear muscle contraction on DPOAE measurements.

2.4. DPOAE generation and measurement

Equilevel primary tones, f_1 and f_2, were generated under computer control using Tucker-Davis System 2 audio processing equipment. More specifically, the computer generated primaries

APPENDIX #5

DAMD17-93-V-3013

Bobbin and Berlin

August 31, 1995 (8:58am)

DRAFT

Chronic low-level noise exposure alters distortion product otoacoustic emissions

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APPENDIX #4

DAMD17-93-V-3013

Bobbin and Berlin

298 The effect of continuous moderate-level noise exposure on contralateral suppression

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Acoustical stimulation of the contralateral ear, with either wideband noise (WBN) or tonal stimuli near the primary frequencies, suppresses the amplitude of both quadratic (f_2-f_1; Kirk and Johnstone, Hear. Res. 67: 20-34, 1993) and cubic ($2f_1$-f_2; Puel and Rebillard, J. Acoust. Soc. Am. 87: 1630-1635, 1990; Kujawa *et al.*, Hear. Res. 68: 97-106, 1993) distortion product otoacoustic emissions (DPOAEs). The suppressive effect of the contralateral acoustic stimulation is mediated by medial olivocochlear (MOC) innervation of the ipsilateral outer hair cells (OHCs). The MOC may be involved in the effects of noise exposure on the cochlea. Therefore, the purpose of this investigation was to test the hypothesis that chronic exposure to a continuous moderate-level narrowband noise (NBN; 1.025-2.125 kHz; 85 dB SPL) affects the amount of contralateral suppression in both the f_2-f_1 and $2f_1$-f_2 DPOAE.

Thirty five pigmented guinea pigs (anesthetized with urethane and middle ear muscles sectioned) were grouped according to the number of days exposed to the NBN (days 0, 1, 3, 5, 7, 9, 11; n = 5). DPOAEs were recorded, both with and without a WBN (0.9 - 15.7 kHz; 70 dB SPL) presented to the contralateral ear, for several combinations of f_1 and f_2 (f_2-f_1=1.2; L_1=L_2=60 dB SPL).

Results reveal that, in general, the f_2-f_1 DPOAE is more sensitive to the effects of contralateral stimulation than its $2f_1$-f_2 counterpart. In addition, while there appeared to be a trend toward a greater amount of suppression for an increasing amount of noise exposure for certain f_2-f_1 DPOAEs, this trend was not apparent in any $2f_1$-f_2 DPOAEs. A significant increase ($p < 0.05$) in the amount of contralateral suppression occurred between day 11 and day 0 animals when f_2-f_1 was equal to 0.75 kHz (f_1=3.75 kHz, f_2=4.5 kHz).

Our preliminary conclusion is that chronic exposure to a continuous moderate-level NBN may increase the amount of contralateral suppression of the f_2-f_1 but not the $2f_1$-f_2 DPOAE. Future experiments will examine this phenomena in greater detail.

NIH (P01DC00379, R01DC00722, T32-DC00007), DAMD 17-93-V-3013, AASERT DAAH0494G0111, Kam's Fund for Hrg. Res., Louisana Lions Eye Fnd.

Abstracts of the 18th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 298, Feb 5-9, 1995

APPENDIX #3

DAMD17-93-V-3013

Bobbin and Berlin

297 Changes in distortion product otoacoustic emissions following continuous noise exposure

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Exposure to chronic moderate level interrupted noise will "toughen" chinchilla ears such that the amplitude of distortion product otoacoustic emissions (DPOAEs) decrease during the first few days of exposure and then begin to recover (Subramaniam *et al.*, Hear. Res. 74: 204-216, 1994). Since otoacoustic emissions reflect outer hair cell (OHC) activity, OHCs are the most likely mediators of this toughening effect. We tested whether a chronic continuous noise would produce this toughening effect in guinea pigs.

In a sound attenuated chamber, guinea pigs were exposed to a moderate level (85 dB SPL) band of noise (cut offs at 1.025 and 2.125 kHz) for 0, 1, 3, 5, 7, 9 and 11 days. Two different DPOAEs (i.e., f_2-f_1 and $2f_1$-f_2) were used as a monitor of toughening. The f_1 and f_2 equilevel primary tones were calculated to maintain an f_2/f_1 ratio of 1.2 to produce the following DPOAEs: $2f_1$-f_2 at 1, 1.25, 2, 3, 4, 5, 6 and 8kHz; and f_2-f_1 at 0.312, 0.5, 0.75, 1, 1.25, 1.5 and 2kHz. The intensity of the primaries ranged from 25 to 70 dB SPL.

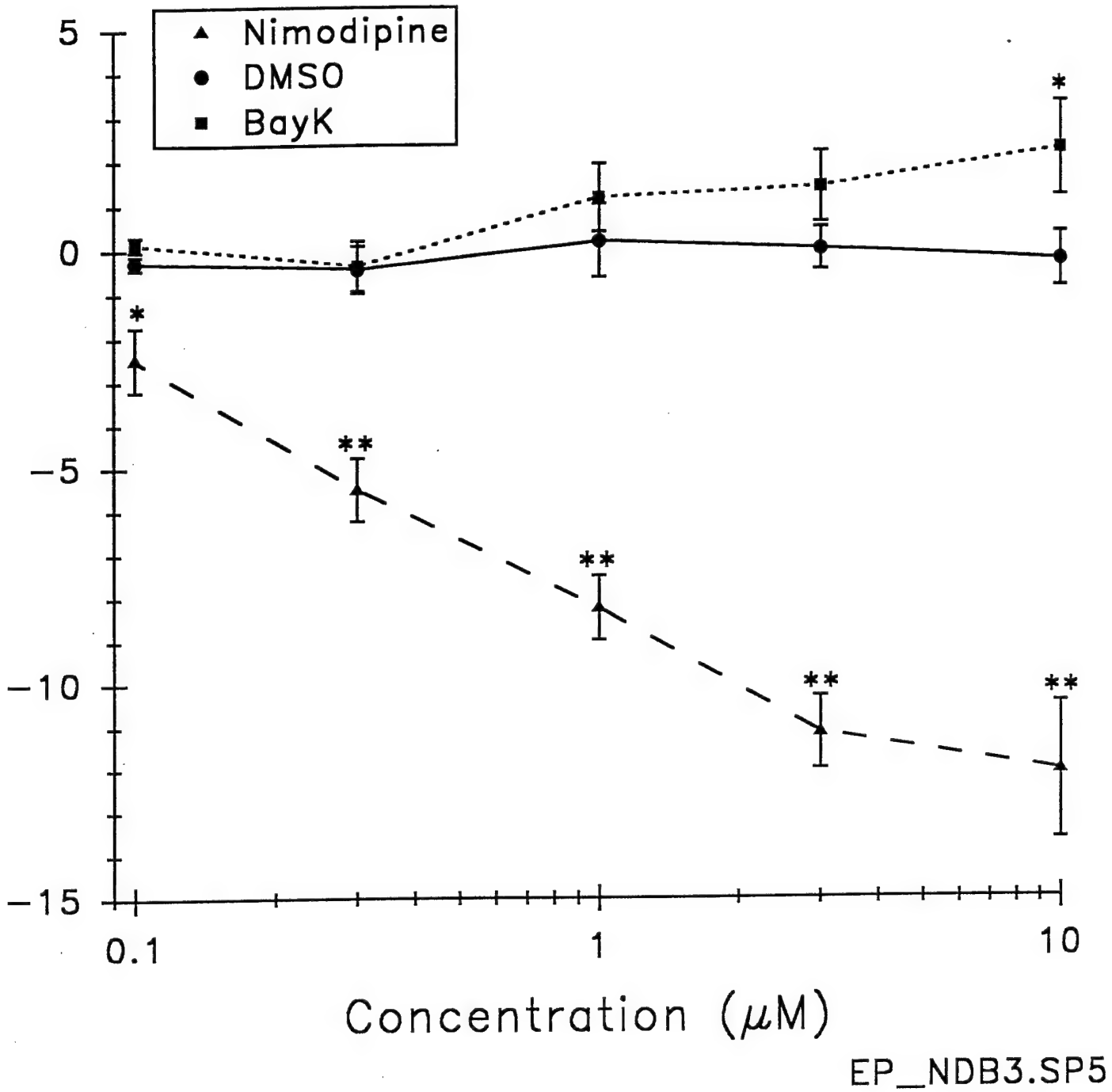
The chronic continuous noise reversibly suppressed DPOAE amplitudes. Suppression was maximum on day 3 and returned towards initial values during days 5, 7, 9 and 11. For $2f_1$-f_2 recovery was complete at 3k, 4k, 5k, 6k, and 8k, but not at lk, 1.25k, and 2k. For f_1-f_2 amplitudes the amount of recovery was less.

Results indicate that toughening does occur in guinea pigs in response to the chronic continuous noise exposure. In addition, it appears that f_2-f_1 is a more sensitive indicator of noise effects on the cochlea than $2f_1$-f_2 .

Supported by NIH grants (P01-DC00379, R01-DC00722), DAMD 17-93-V-3013, Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation

Abstracts of the 18th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, Abstract 297, Feb 5-9, 1995.

Fig. 8


Nimodipine
DMSO
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Δ EP (mV)
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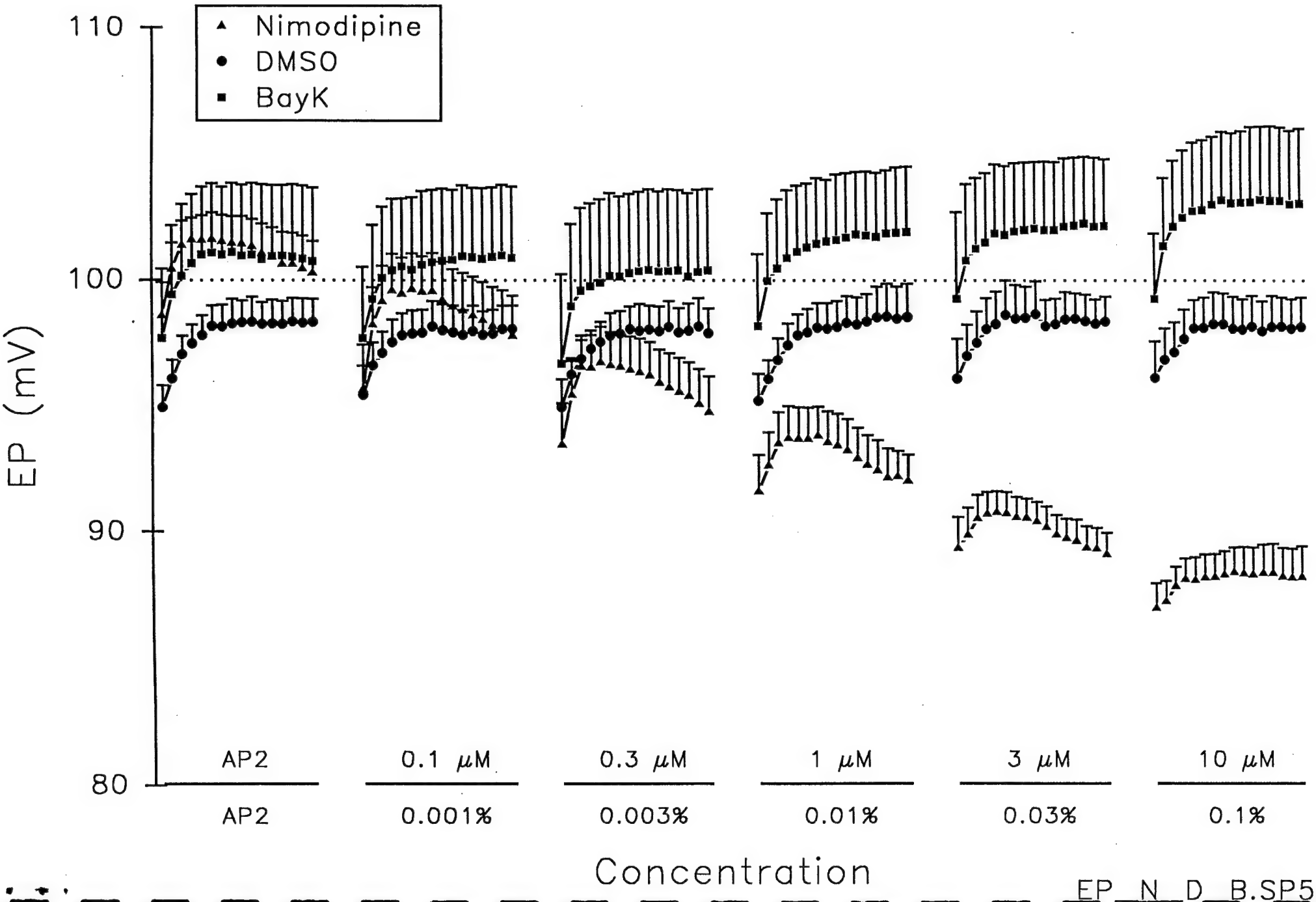
Fig 7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were sent to two separate channels of a DA1 Digital-to-Analog Converter and attenuated to desired levels using PA4 Programmable Attenuators. The analog signals were then sent to two separate channels of an FT5 Anti-aliasing Low-pass Filter with a 20 kHz cutoff frequency and then to an HB5 Headphone Buffer before being sent to two separate speakers (Etymotic Research, ER-2) housed within an acoustic probe assembly. The acoustic probe assembly was tightly coupled to the right ear of each animal. DPOAEs were detected by a sensitive microphone (Etymotic Research, ER-10) also housed within the probe assembly and amplified using a microphone preamplifier (Etymotic Research, ER-1072). A dynamic signal analyzer (Hewlett-Packard, 3561A) was used to average the DPOAE responses for FFT analysis and spectral display (CF = DPOAE frequency; span = 1 kHz; BW = 3.75 Hz).

DPOAE amplitudes were measured for several combinations of f_1 and f_2 having a ratio f_2/f_1=1.2 (Table I). This ratio is within the range describing the optimal frequency separation of f_1 and f_2 for the guinea pig (1.2-1.3; Brown, 1987; Brown and Gaskill, 1990). Since the place of origin of the DPOAEs is believed to be near, or at, the f_2 place on the cochlear partition (Matthews and Molnar, 1986; Brown et al., 1992; Allen and Fahey, 1993), all data are expressed as a function of f_2 rather than the frequency of the DPOAE (Table I). DPOAEs were elicited with equilevel primaries tones (L_1=L_2). The primary tones were presented in descending order, starting at a level of 70 dB SPL and decreasing in 5 dB steps to 20 dB SPL. Both quadratic (f_2-f_1) and cubic ($2f_1$-f_2) DPOAEs were studied. DPOAE I/O functions [i.e., plots of primary level vs. DPOAE amplitude for each f_2] and DPOAE audiograms [i.e., f_2 vs. DPOAE amplitude] were generated.

2.5. Time-varying amplitude alteration measures

The procedures used to investigate the influence of continuous, moderate-level (L_1=L_2=60 dB SPL) primary stimulation on the f_2-f_1 DPOAE at 1.25 kHz (f_1=6.25 kHz; f_2=7.5 kHz) have been previously described by Kujawa et al. (1995). Briefly, after a 15 min rest period from acoustic stimulation, 100 consecutive 10-spectra averages of the DPOAE amplitude were measured during continuous primary stimulation. These 100 averages required approximately 500 s (8.3 min) of continuous stimulation (5 s/average). Next, a one min rest from primary stimulation was given. Following this rest, the primaries were again turned on and 40 consecutive 10-spectra averages of the DPOAE amplitude were measured. The 40 averages required approximately 200 s (2.3 min) of continuous stimulation.

2.6. Contralateral noise generation

The 70 dB SPL wideband noise (WBN) delivered to the contralateral (left) ear was also generated under computer control using Tucker-Davis System 2 audio processing equipment. In particular, a W1 Waveform Generator was set up to produce a uniformly distributed noise signal. The level of the was

controlled with a PA4 Programmable Attenuator and then sent to a speaker (Etymotic Research, ER-2). A polyethylene tube (1.35 mm i.d., 292 mm length) attached to the output of the speaker was used to deliver the noise down the hollow ear bar coupled to the entrance of the bony canal of the left ear. The spectrum of the noise presented to the contralateral ear was flat (±10 dB) from 0.9-15.8 kHz and rolled off approximately 50 dB/octave above 15.8 kHz.

2.7. Contralateral suppression measurements

DPOAEs were recorded, both with and without the WBN (70 dB SPL) presented to the contralateral ear for several combinations of f_1 and f_2 (f_2/f_1=1.2; L_1=L_2=60 dB SPL). Table I lists the primary frequencies and the corresponding DPOAEs (f_2-f_1 and $2f_1$-f_2) that were studied. The paradigm used to measure the amount of contralateral suppression is the same as that used by Kujawa et al. (1995). Briefly, five consecutive averages of DPOAE amplitude (10 spectra per averages) were first obtained in the absence of contralateral stimulation. The WBN was then delivered to the contralateral ear and again 5 consecutive 10-spectra averages of the DPOAE amplitude were obtained. The final 5 consecutive averages were taken after the contralateral stimulation was removed. Each of these averages required approximately 5 seconds to complete. A total of 25 seconds of amplitude monitoring was obtained for each condition.

2.7. Data analysis

Data are presented as means ± S.E. Effects of the low-level noise exposure on DPOAE I/O functions were quantified using one-way (by exposure group) analysis of variance (ANOVA) and Newman-Keuls post hoc tests. Effects of the low-level noise exposure on the time-varying amplitude alterations of the f_2-f_1 DPOAE were quantified using repeated measures analysis of variance (ANOVA) and Newman-Keuls post hoc tests. The effect of the number of days of conditioning noise exposure on contralateral suppression of DPOAEs were quantified using one-way analysis of variance (ANOVA) and Student-Newman-Keuls post-hoc tests. The Kruskal-Wallis One-Way ANOVA on Ranks test was used in cases where the assumptions for parametric tests were violated. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of continuous low-level noise exposure on DPOAE I/O functions

Fig. 2 illustrates the $2f_1$-f_2 DPOAE I/O functions of the unexposed (control) guinea pigs and the guinea pigs exposed continuously to the 65 dB SPL NBN for either 3 or 11 days. The results show significant differences between the control group and both noise-exposed groups in the DPOAE I/O functions elicited when f_2 was in the region of the noise band (Fig. 2; f_2 = 1.5 kHz

and 1.875 kHz). Only intensity levels ranging from 40 to 55 dB SPL were significantly different (Fig. 2). All other DPOAE I/O functions were not significantly different (Fig. 2; f_2 = 3.0, 4.5, 6, 7.5, 9, and 12 kHz; $p > 0.05$). The $2f_1-f_2$ DPOAE audiogram shown in Fig. 3 further demonstrates the frequency-specific, and very localized nature of the effect of the low-level noise. DPOAE amplitudes were significantly reduced in both noise-exposed groups as compared with the unexposed group only when f_2 fell within the frequency region of the noise band (f_2 = 1.5 kHz and 1.875 kHz; $p < 0.01$).

Fig. 4 illustrates the f_2-f_1 DPOAE I/O functions of the unexposed (control) guinea pigs and the guinea pigs exposed continuously to the 65 dB SPL NBN for either 3 or 11 days. The results do not show any significant differences in the DPOAE I/O functions between any of the groups tested for any combination of primary tones studied ($p > 0.05$). However, there appears to be a slight trend, especially for the DPOAEs measured in animals exposed for 3 days, to have smaller amplitudes than normal when the DPOAE frequency fell within the noise band (Fig. 4; f2=6, 7.5, and 9 kHz). The f_2-f_1 DPOAE audiogram shown in Fig. 5 provides another representation of the data shown in Fig. 4 when the level of the primary tones was 45 dB SPL.

3.2. Effect of continuous low-level noise exposure on the time-varying amplitude alterations of the f_2-f_1 DPOAE

Fig. 6 illustrates the time-varying amplitude alterations of the f_2-f_1 DPOAE at 1.25 kHz (f_1=6.25 kHz; f_2=7.5 kHz; $L_1=L_2$=60 dB SPL) for the control group and the groups exposed to the 65 dB SPL narrowband noise continuously for 3 and 11 days. No significant differences were found in the time-varying amplitude alterations for any of the groups tested. In addition, the overall shape of the curves were not altered in any way by the noise exposure and all curves followed the pattern of change described by Kujawa et al.(1995).

3.3. Effect of continuous low-level noise exposure on contralateral suppression

Fig. 7 and Fig. 8 illustrate the average (n=13 animals/group) amount of contralateral suppression measured in the unexposed group of animals and the groups of animals exposed to the 65 dB SPL narrowband noise for both the $2f_1-f_2$ and f_2-f_1 DPOAE. We were unable to demonstrate any systematic changes in the amount of contralateral suppression over the course of the continuous noise exposure in either the f_2-f_1 or $2f_1-f_2$ DPOAEs.

4. Discussion

The principal finding of this study was the demonstration of the frequency-dependent and very localized reductions in the $2f_1$-f_2 DPOAE I/O functions as a result of exposure to continuous, low-level narrowband noise. This result may be a functional measure of the changes in OHC response to ATP reported by Chen et al. (1995).

Significant amplitude reductions of the $2f_1$-f_2 DPOAE occurred in both noise exposed groups only when the frequency of f_2 was within the noise exposure band. This finding is consistent with the belief that the f_2 place along the cochlear partition is the generation site of DPOAEs (Matthews and Molnar, 1986; Brown et al., 1992; Allen and Fahey, 1993). Damage to this place results in the reduction and possibly complete loss of the corresponding DPOAE (Siegel and Kim, 1982a,b; Zurek et al., 1982). Further, the changes in DPOAE amplitude induced by the noise exposure occurred only in the region of the I/O functions thought to be mediated by active cochlear mechanics. Low-to-moderate level primaries are used to elicit the DPOAEs within this region. These DPOAEs are physiologically vulnerable to the same factors (e.g., noise exposure and ototoxicity) that normally damage or destroy OHC function (Kim, 1980; Zurek et al., 1982; Brown et al., 1989; Mills et al., 1993; Subramaniam et al., 1994). The generation of DPOAEs elicited by high-level primaries, on the other hand, is attributed to the passive mechanical properties of the cochlea. These emissions are relatively invulnerable to cochlear insult (Zurek et al., 1982) and can even be measured in the ear of a dead animal (Schmiedt and Adams, 1980; Pers. observ.). This is also reflected in our results.

It is interesting to note that there were similar reductions in DPOAE amplitude for the groups of animals exposed for 3 and 11 days. This pattern of amplitude change resembles that of an ATS. There are no previous reports of "ATS-like" changes in DPOAE amplitude in response to long-term noise exposure. However, since we only monitored the changes in DPOAE amplitude on two days during our noise exposure protocol, more experiments will have to be performed to better characterize the pattern of DPOAE amplitude change over the course of a continuous noise exposure.

Our results did not reflect any alterations in efferent activity as a result of the chronic noise exposure. Contra-lateral sound suppression is thought to be mediated by medial olivocochlear (MOC) neurons which synapse directly with ipsilateral outer hair cells (OHCs; Warren and Liberman, 1989). This efferent innervation influences the response characteristics of the OHCs which in turn result in alterations in the mechanical response of the cochlear partition to sound. These alterations

are reflected as changes (decreases) in DPOAE amplitude (Puel and Rebillard, 1990; Kirk and Johnstone, 1993; Kujawa et al., 1993, 1995). We found no systematic changes in the amount of contralateral suppression over the course of the noise exposure for either the f_2-f_1 or $2f_1$-f_2 DPOAEs. It is possible that the paradigm that we used to measure contralateral suppression was not suitable for detecting such changes. The level of the primary tones (60 dB SPL) used to elicit the DPOAEs for the suppression experiments was above that mediated by active cochlear processes. This fact alone may have precluded us from measuring noise-induced alterations in the amount of contralateral suppression since at 60 dB SPL we did not measure a difference in DPOAE amplitude between the control and the noise-exposed animals.

We also did not find significant differences in the time-varying amplitude characteristics of the f_2-f_1 DPOAE between the unexposed group of animals and the noise-exposed groups. The overall shape of the curves were not altered in any way by the noise exposure and all curves followed the pattern of change described by Kujawa et al.(1995).

In summary, results of this study show that chronic low-level noise exposure does, in fact, alter cochlear function as measured by changes in the $2f_1$-f_2 DPOAE I/O functions. However, significant noise-induced changes were not found in the f_2-f_1 DPOAE I/O functions. The DPOAE amplitude changes observed resembled the changes in sensitivity associated with an ATS. Evidence of DPOAE amplitudes alterations lends support to the findings of Chen et al. (1995) which suggests that chronic low-level noise exposure induces metabolic changes in the OHCs, possibly due to alterations in the number of ATP receptor proteins (i.e., up-regulation or down-regulation) on the hair cells. The time-varying amplitude alterations of the f_2-f_1 DPOAE and the amount of contralateral suppression were unaffected by chronic exposure to the low-level noise. It is possible that the paradigms used to test for the effects of the noise exposure on these measures precluded us from seeing noise-induced changes.

Acknowledgements

The authors wish to thank David Owens for writing the computer programs used to run our signal processing equipment and Sharon G. Kujawa for her advice. Supported by DAMD 17-93-V-3013, NIH grant R01-DC00722, Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation.

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Figure Legends

Fig. 1. The spectrum of the 65 dB SPL (A-scale) exposure noise as viewed on the Hewlett-Packard 3561A dynamic signal analyzer. The x-axis represents frequency (in Hz) with each division being equal to 1000 Hz. The y-axis represents the amplitude of the signal (in dBV) with each division being equal to 10 dBV. Cutoff frequencies are 1100 and 2000 Hz.

Fig. 2. Effect of continuous low-level noise exposure on cubic ($2f_1$-f_2) DPOAE I/O functions. Data are represented as means ± S.E. (n=13/group) for the unexposed group (0 day; open circles), the group of animals exposed for 3 days (closed circles), and the group of animals exposed for 11 days (open triangles). One asterisk indicates a statistically significant difference at the 0.5% level ($p<0.05$) and two asterisks indicates a statistically significant difference at the 0.1% level ($p<0.01$). The dashed line represents the noise floor.

Fig. 3. $2f_1$-f_2 DPOAE audiogram. DPOAE amplitudes plotted as a function of f_2 when the level of the primary tones (L_1=L_2) was 45 dB SPL. Data are represented as means ± S.E. (n=13/group) for the unexposed group (0 day; open circles), the group of animals exposed for 3 days (closed circles), and the group of animals exposed for 11 days (open triangles). One asterisk indicates a statistically significant difference at the 0.5% level ($p<0.05$) and two asterisks indicates a statistically significant difference at the 0.1% level ($p<0.01$). NF designates the average noise floor (mean = solid line; s.e. = dashed line) as a function f_2 (kHz).

Fig. 4. Effect of continuous low-level noise exposure on quadratic (f_2-f_1) DPOAE I/O functions. Data are represented as means ± S.E. (n=13/group) for the unexposed group (0 day; open circles), the group of animals exposed for 3 days (closed circles), and the group of animals exposed for 11 days (open triangles). The dashed line represents the noise floor.

Fig. 5. f_2-f_1 DPOAE audiogram. DPOAE amplitudes plotted as a function of f_2 when the level of the primary tones (L_1=L_2) was 45 dB SPL. Data are represented as means ± S.E. (n=13/group) for the unexposed group (0 day; open circles), the group of animals exposed for 3 days (closed circles), and the group of animals exposed for 11 days (open triangles). NF designates the average

noise floor (mean = solid line; s.e. = dashed line) as a function f_2 (kHz).

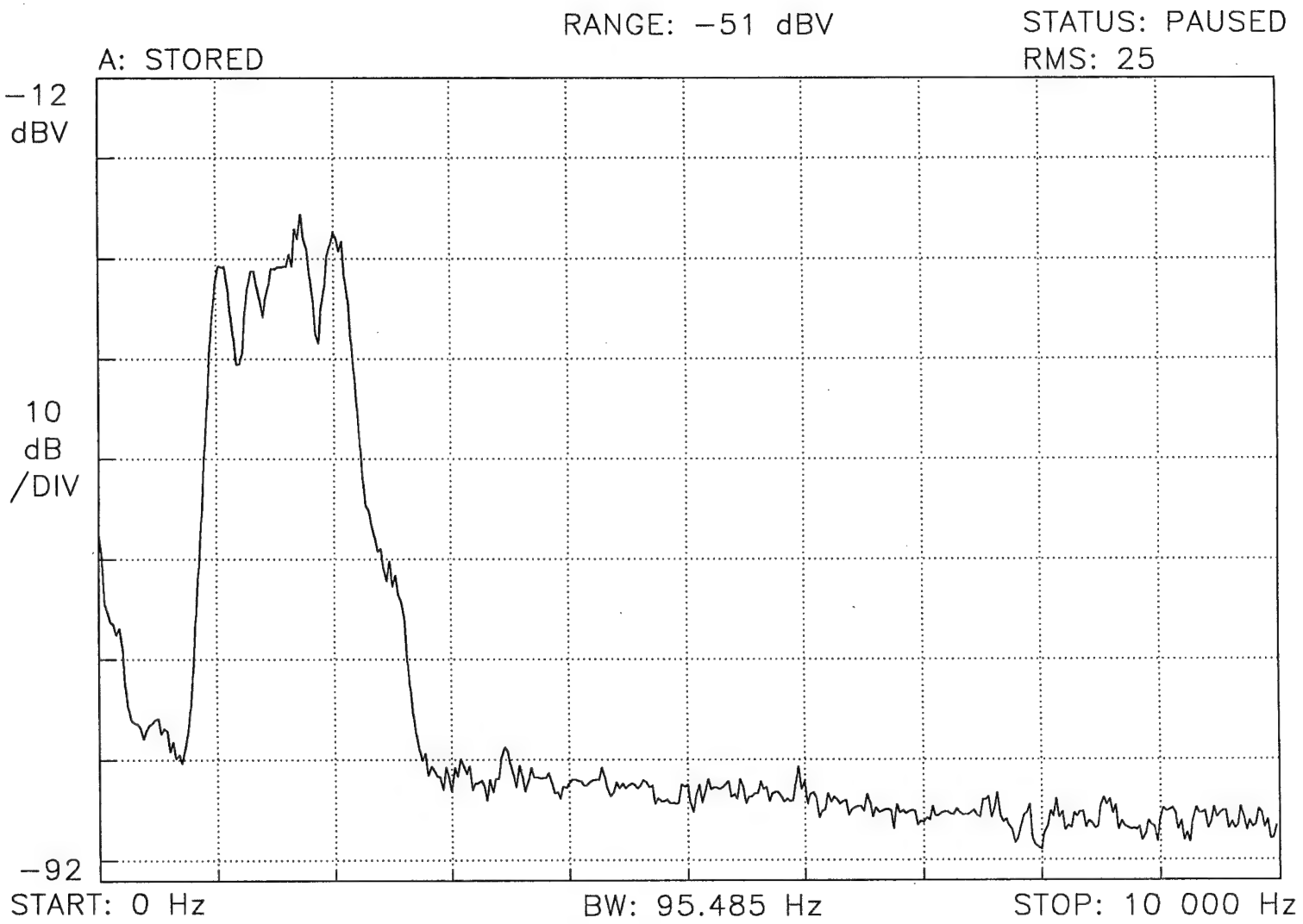
Fig. 6. Effect of continuous low-level noise exposure on the time-varying amplitude characteristics of the f_2-f_1 DPOAE (f_1=6.25 kHz, f_2=7.5 kHz, L_1=L_2=60 dB SPL). Data are represented as means (n=13 animals/group, 2 measurements/animal) for the unexposed group (0 day; open circles), the group of animals exposed for 3 days (closed circles), and the group of animals exposed for 11 days (open triangles). Symbols are plotted as the mean of two consecutive 10 spectra averages each requiring 5 s to complete. The break in the trace (C-D) represents the 1 min rest period from primary stimulation. Points A-F were used to calculate the magnitudes of the amplitude changes, slopes of suppression, and recovery functions so that statistical analyses may be performed.

Fig. 7. Effect of continuous low-level noise exposure on the amount of contralateral suppression for the $2f_1$-f_2 DPOAE. DPOAEs were monitored before (5 trials), during (5 trials), and after (5 trials) presentation of WBN (70 dB SPL) to the contralateral ear. Each trial represents a 10 spectra average and required 5 s to complete. The bars represent the amount of contralateral suppression which was calculated by subtraction the mean of the 5 "during" trials from the mean of the 5 "before" trials. Data are represented as means ± S.E. (n=13).

Fig. 8. Effect of continuous low-level noise exposure on the amount of contralateral suppression for the f_2-f_1 DPOAE. DPOAEs were monitored before (5 trials), during (5 trials), and after (5 trials) presentation of WBN (70 dB SPL) to the contralateral ear. Each trial represents a 10 spectra average and required 5 s to complete. The bars represent the amount of contralateral suppression which was calculated by subtraction the mean of the 5 "during" trials from the mean of the 5 "before" trials. Data are represented as means ± S.E. (n=13). The asterisk indicates a statistically significant difference at the 0.5% level ($p<0.05$).

Table 1. Primary Tones and Corresponding DPOAEs (measured in kHz)

f_1	f_2	(f_2-f_1)	$2f_1-f_2$
1.250	1.500	----	1.000
1.563	1.875	0.312	1.250
2.500	3.000	0.500	2.000
3.750	4.500	0.750	3.000
5.000	6.000	1.000	4.000
6.250	7.500	1.250	5.000
7.500	9.000	1.500	6.000
10.000	12.000	2.000	8.000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dB
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STOP: 10 000 Hz

Figure 1

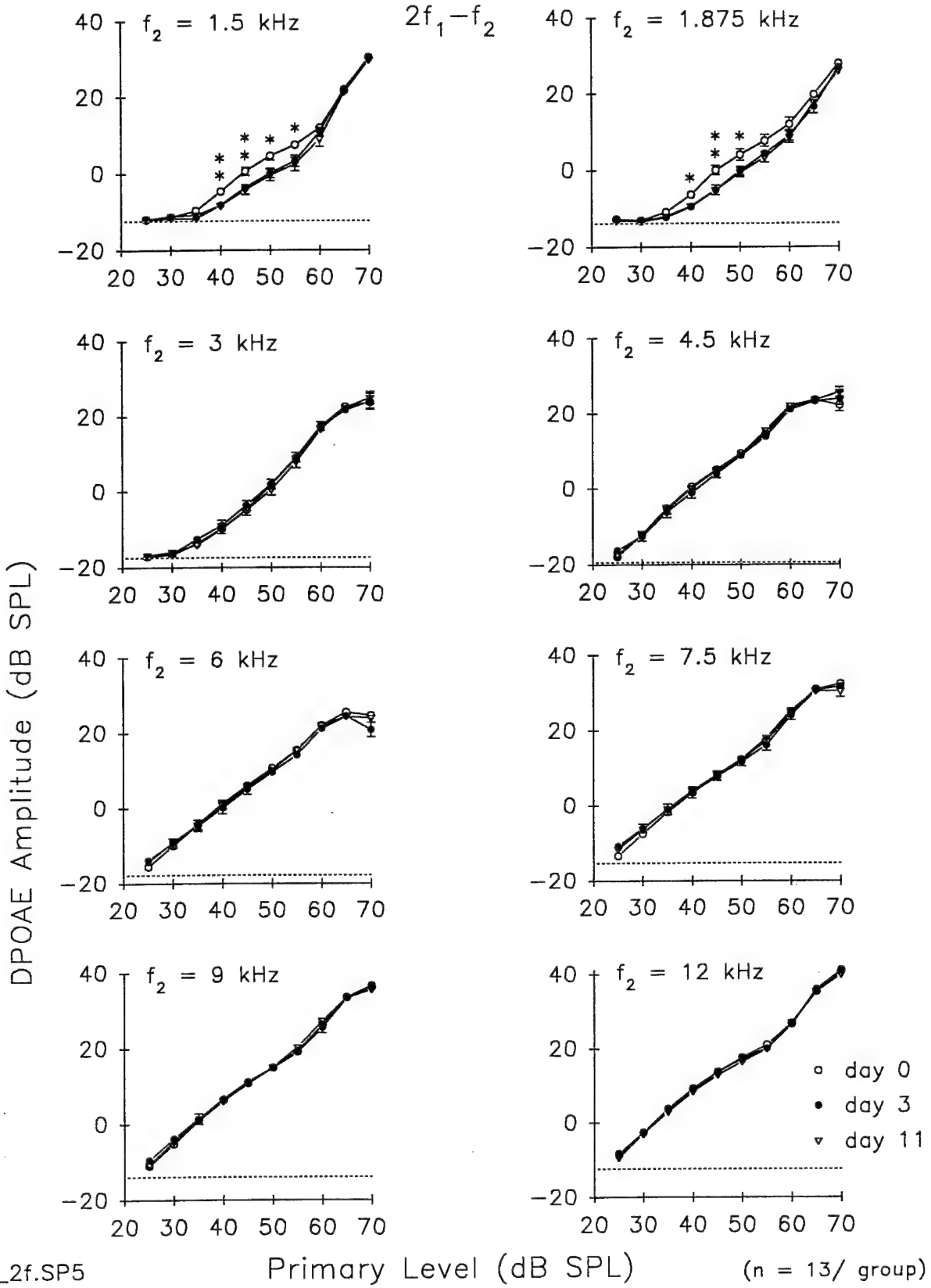

2f1−f2
f2 = 1.5 kHz
f2 = 1.875 kHz
f2 = 3 kHz
f2 = 4.5 kHz
f2 = 6 kHz
f2 = 7.5 kHz
f2 = 9 kHz
f2 = 12 kHz
40
20
0
−20
20 30 40 50 60 70
DPOAE Amplitude (dB SPL)
Primary Level (dB SPL)
day 0
day 3
day 11
R_10_2f.SP5
(n = 13/ group)

Figure 2

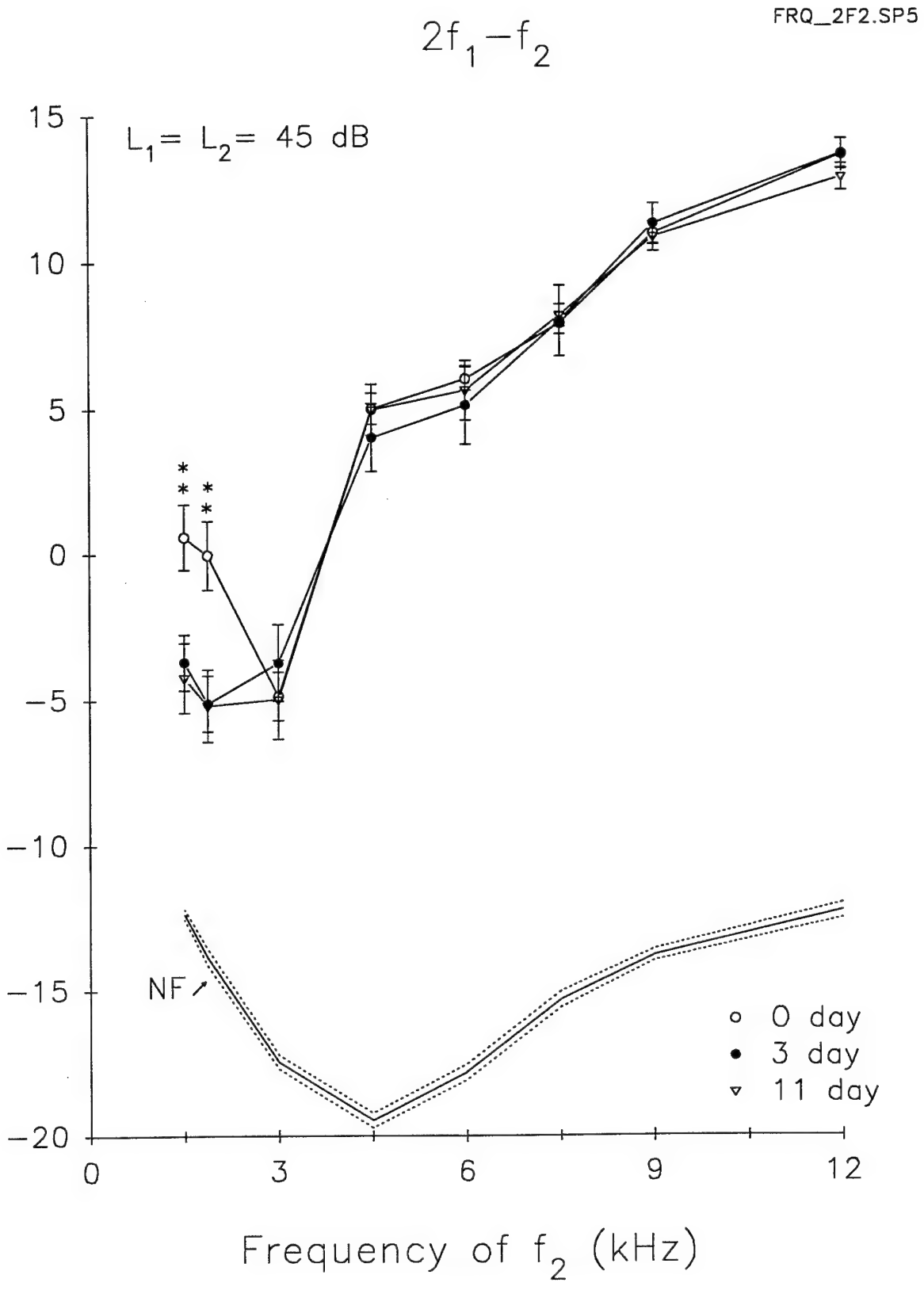

FRQ_2F2.SP5
$2f_1 - f_2$
$L_1 = L_2 = 45$ dB
15
10
5
0
−5
−10
−15
−20
0
3
6
9
12
NF
0 day
3 day
11 day
Frequency of f_2 (kHz)

Figure 3

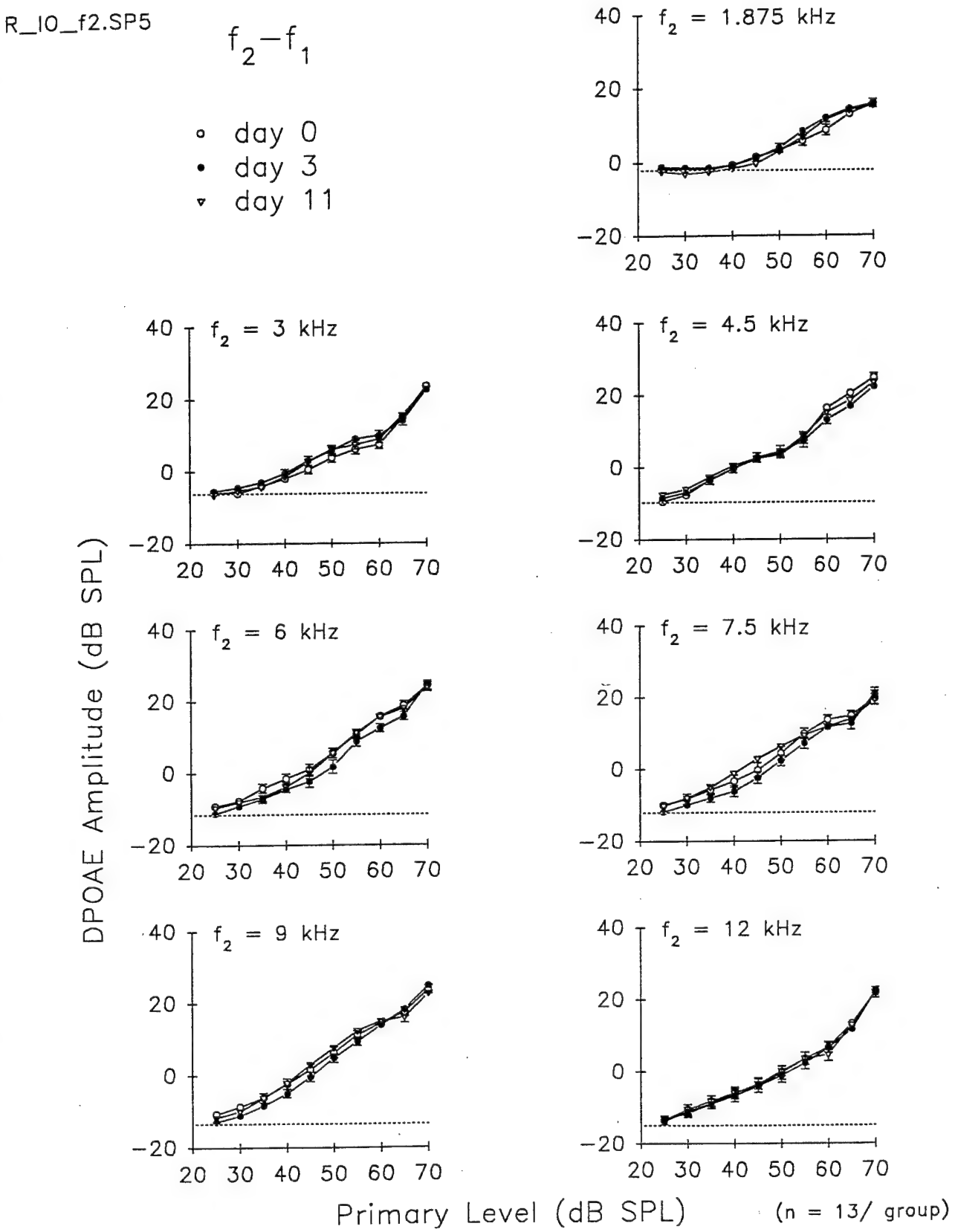

R_10_f2.SP5
$f_2 - f_1$
day 0
day 3
day 11
f_2 = 1.875 kHz
f_2 = 3 kHz
f_2 = 4.5 kHz
f_2 = 6 kHz
f_2 = 7.5 kHz
f_2 = 9 kHz
f_2 = 12 kHz
DPOAE Amplitude (dB SPL)
Primary Level (dB SPL)
(n = 13/ group)

Figure 4

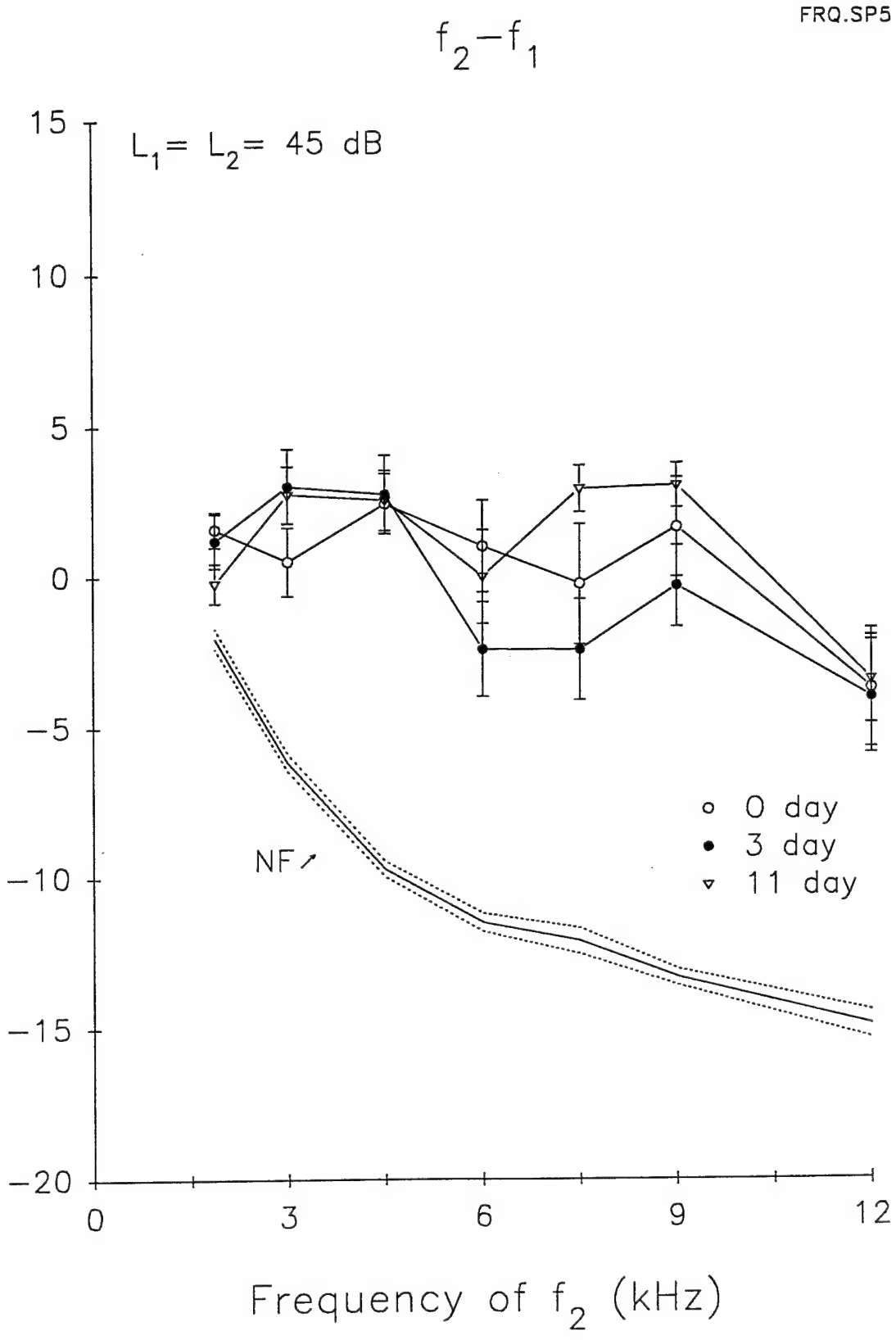

FRQ.SP5
$f_2 - f_1$
$L_1 = L_2 = 45$ dB
15
10
5
0
−5
−10
−15
−20
0
3
6
9
12
NF
0 day
3 day
11 day
Frequency of f_2 (kHz)

Figure 5

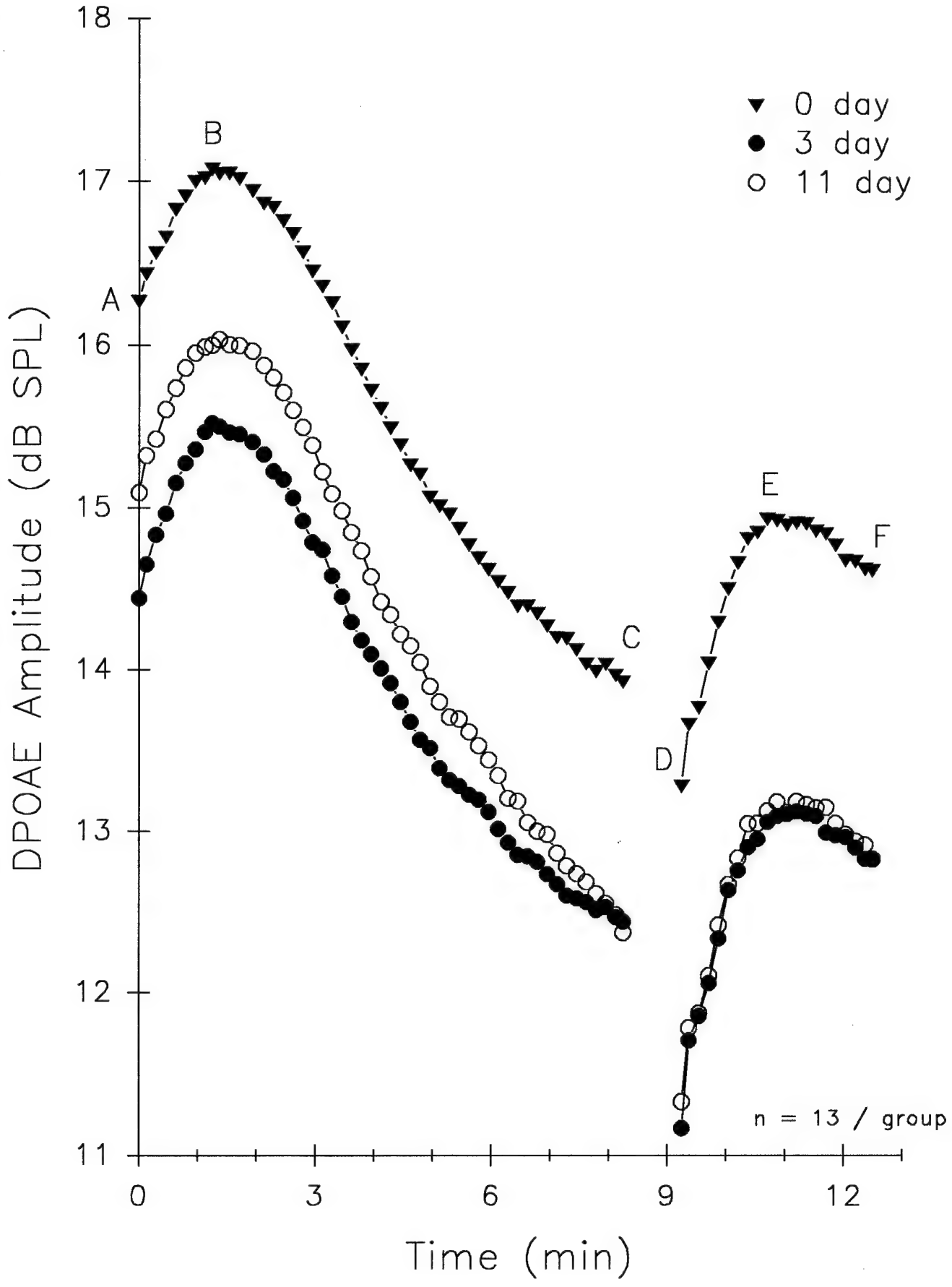
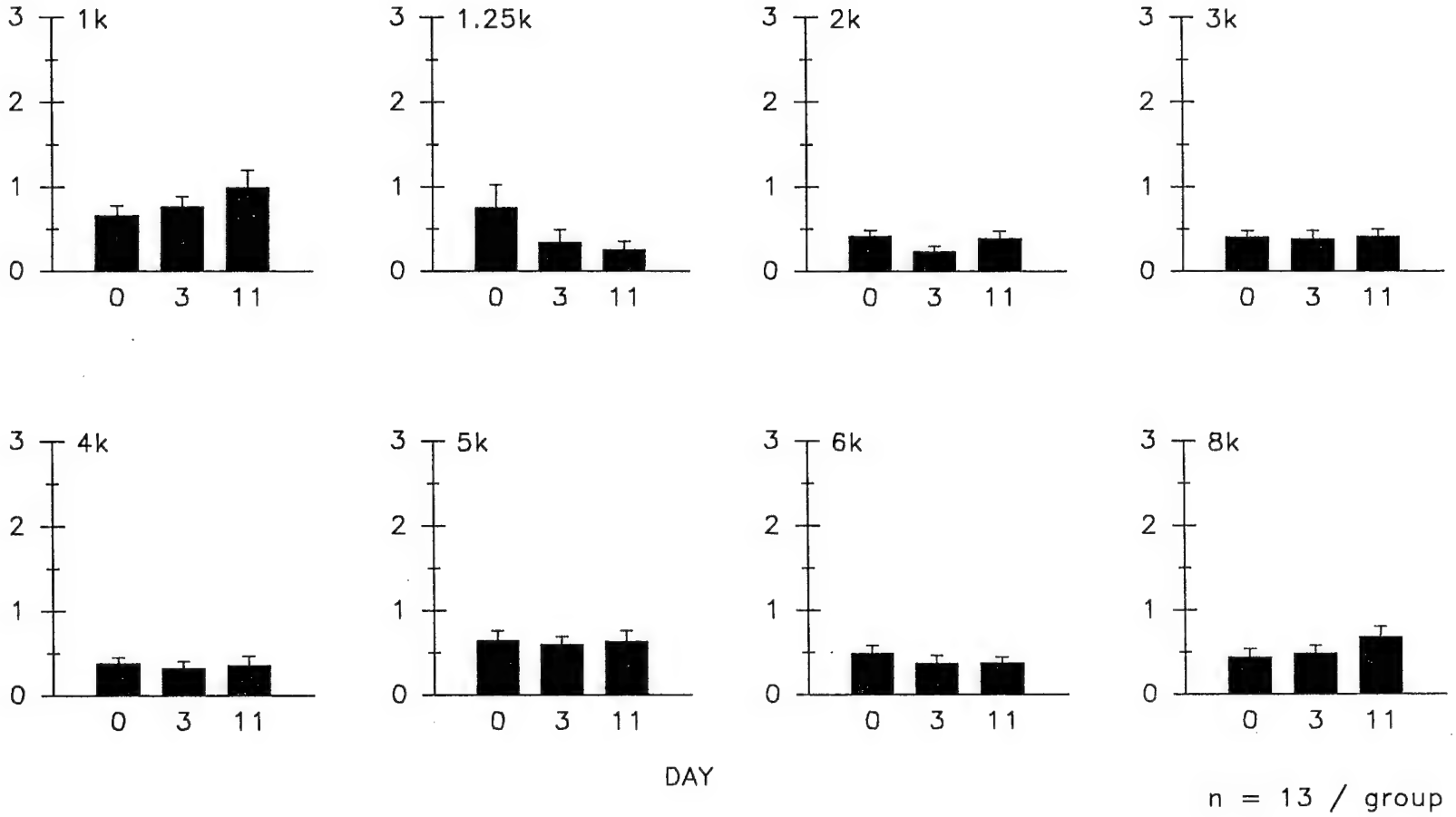

18
17
16
15
14
13
12
11
DPOAE Amplitude (dB SPL)
0
3
6
9
12
Time (min)
A
B
C
D
E
F
0 day
3 day
11 day
n = 13 / group

Figure 6

R_C_ST2.SP5

difference between the mean of the first five no noise values and the mean of the five values with contra noise

$2f_1 - f_2$



n = 13 / group

Figure 7

$f_2 - f_1$

R_C_ST.SP5

difference between the mean of the first five no noise values and the mean of the five values with contra noise

n = 13 / group

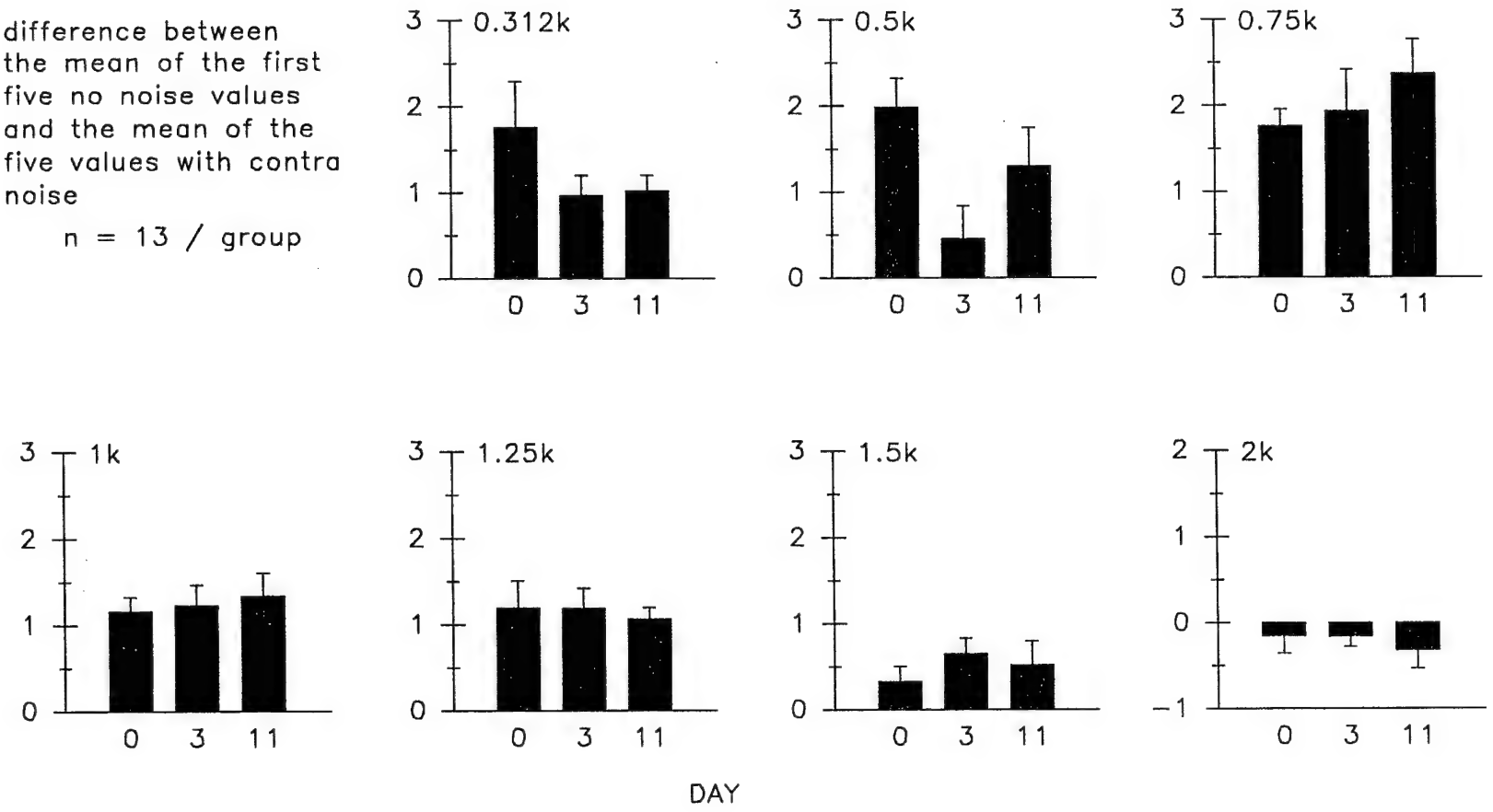


Figure 8

APPENDIX #6
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HEARING RESEARCH

Noise exposure alters the response of outer hair cells to ATP

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Received 8 March 1995; revised 9 March 1995; accepted 16 March 1995

Abstract

The outer hair cells (OHCs) are one target of noise-induced effects. To date there are few studies which examine changes in the function of OHCs induced by noise exposure. There is increasing evidence that ATP may be a neuromodulator acting on OHCs. Therefore, we examined the possibility that the response to ATP may be altered by low-level noise exposure. ATP was tested on cation currents recorded from outer hair cells (OHCs) isolated from chronic noise-exposed guinea pigs and compared to currents recorded from normal control animals. The whole-cell variant of the patch-clamp technique was used. The incidence of response to 100 μM ATP was decreased in OHCs from noise-exposed animals as compared to controls when normal internal and external solutions were employed. When K^+ was substituted by *N*-methyl-glucamine (NMG^+) in the pipette solution, there were significant differences in the magnitudes of ATP-evoked currents between cells from noise-exposed and control animals. This was observed in both normal and 20 mM Ba^{2+} external solutions. In addition, the response to ATP exhibited a dependency on OHC length. In short OHCs (< 65 μm) from noise-exposed animals the magnitude of the response to ATP was significantly reduced. By contrast, the response in long OHCs (> 65 μm) from noise-exposed animals was increased. Results suggest that low-level noise exposure induces changes in OHCs which affect the response of the cell to ATP.

Keywords: Voltage-clamp; Patch-clamp; ATP-gated channel; Cochlea; Noise exposure

1. Introduction

Noise exposure induces several alterations in the structure and function of the cochlea (see review by Saunders et al., 1985). The outer hair cells (OHCs) are one target of noise-induced effects (e.g., Cody and Russell, 1985; Puel et al., 1988; Decory et al., 1991; Franklin et al., 1991; Boettcher et al., 1992; Subramaniam et al., 1994). Morphological evidence shows that noise exposure causes: damage to OHC stereocilia, changes in intracellular structures such as mitochondria, and swelling or loss of the OHCs (Saunders et al., 1985). Some of these morphological changes have been correlated with changes in neuronal thresholds (Boettcher et al., 1992) and distortion product otoacoustic emissions (DPOAEs). The latter are thought to reflect the physiological state of the OHCs (Subramaniam et al., 1994).

There are only a few studies which examined the effect of intense sound on the function of the hair cells directly. Decory et al. (1991) showed that isolated OHCs taken from noise-exposed guinea pigs exhibited altered motility and viability. Acoustical-evoked receptor potentials recorded from both inner hair cells (IHCs) and OHCs are altered during and after exposure to intense sound with the OHCs undergoing a sustained depolarization (Cody and Russell, 1985). Some of these effects are probably due to excessive passage of K^+ and Ca^{2+} through the transduction channels of the hair cells together with entrance of Ca^{2+} and Na^+ through voltage-dependent channels. In other systems, it is well known that an increase in the levels of intracellular free Ca^{2+} can initiate events which result in the alteration or death of cells (Berridge, 1994; Morley et al., 1994). Thus there is evidence that the OHCs are excessively depolarized by noise exposure, resulting in altered function and possibly cell death.

There are a host of molecular events occurring in and around OHCs that could be altered by noise exposure. One possibility is an altered response to neurotransmitter or neuromodulator. Bobbin and Thompson (1978) first proposed ATP as a neuromodulator or transmitter in the

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SSDI 0378-5955(95)00115-8

mammalian cochlea (see review Eybalin, 1993). Aubert et al. (1994,1995) present data suggesting that ATP may act as a neuromodulator in vestibular organs. ATP and analogues applied into the perilymph compartment exert profound effects on cochlear function, as indicated by an abolishment of compound action potential of the auditory nerve and DPOAEs together with a shift in the summating potential (Bobbin and Thompson, 1978; Kujawa et al., 1994a). Incubation of organ of Corti with ATP agonist analogs increases inositol phosphate accumulation suggesting an activation of this second-messenger system (Niedzielski and Schacht, 1992). Extracellular application of ATP to isolated OHCs of guinea pig depolarizes the cell membrane by inducing non-selective cation currents (Ashmore and Ohmori, 1990; Nakagawa et al., 1990; Housley et al., 1992; Kujawa et al., 1994a; Nilles et al., 1994). Since antagonists also have profound effects on the cochlear potentials, it appears that endogenous ATP may have a role in ongoing physiological mechanisms (Kujawa et al., 1994b). In addition, noise exposure may increase extracellular levels of ATP to such an extent that it contributes to the excessive depolarization and cell death observed.

Thus we initiated studies of the hypothesis that ATP is involved in noise-induced changes to the OHCs. Specifically, in this study we examined whether the response of OHCs to ATP was altered by a low-level, chronic noise exposure. The intensity of the noise was set below the level thought to induce cellular damage (Bohne, 1976). We recorded the cation currents evoked by ATP in isolated OHCs harvested from chronic noise-exposed guinea pigs and compared them to the currents evoked from control animals using the whole-cell configuration of the patch-clamp technique.

2. Methods

2.1. Noise exposure

Guinea pigs ($n = 35$, in groups of 10 or less; age 1–3 months) were placed in a small sound-attenuating booth and exposed to a continuous, moderate-intensity narrow band noise (cutoffs at 1.1 and 2.0 kHz; A-scale, rms: 65 dB SPL) 24 h a day for 10–11 days. Control guinea pigs ($n = 30$) were maintained in an environment of normal noise at the university's animal care facilities. Both groups of the animals were given free access to food and water during the exposure. The care and use of the animals reported on in this study were approved by the Animal Care and Use Committees of the Louisiana State University Medical Center.

2.2. Cell isolation

OHCs from control and noise exposed guinea pigs were acutely isolated as described previously (Erostegui et al., 1994). Briefly, guinea pigs were anesthetized with pentobarbital (30 mg/kg, i.p.) or urethane (1.5 g/kg, i.p.), decapitated, and the bulla separated and placed in a modified Hank's balanced saline (HBS) (Table 1). The bone surrounding the cochlea was removed and the organ of Corti was placed in a 200 μl drop of HBS containing collagenase (1 mg/ml, Type IV, Sigma) for 5 min. The cells were then transferred into the dishes containing a 100 μl drop of HBS using a microsyringe, and stored at room temperature until use (within about 4 h). The length of each cell was measured with a calibrated reticulum prior to recording. OHCs were selected for study if they met several morphological criteria (Ricci et al., 1994). No morphological changes of isolated OHCs from noise-exposed animals were observed.

Table 1
Composition of solutions (mM)

	Internal		External	
	K^+ sol.	NMG^+ sol.	HBS sol.	Ba^{2+} sol.
NaCl	-	-	137	120
KCl	134	-	5.4	-
$CaCl_2$	0.1	0.5	2.5	-
$BaCl_2$	-	-	-	20
$MgCl_2$	0.5	-	0.5	0.5
CsCl	-	-	-	5
HEPES	5	10	10	10
NMG^+	-	120	-	-
TEA-Cl	-	35	-	-
EGTA	11	11	-	-
Glucose	-	-	10	5
Na_2ATP	2	4	-	-
Na_2GTP	0.1	0.1	-	-
Sucrose	-	10	10	-

HBS: modified Hank's balanced saline; NMG^+: *N*-methyl-glucamine.

2.3. Whole-cell voltage clamp

Single dispersed OHCs either from noise-exposed guinea pigs or normal control animals were voltage clamped using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981) with Axopatch-1D and Axopatch-200A patch-clamp amplifiers (Axon Instruments). Patch electrodes were fabricated from borosilicated capillary tubing (Longreach Scientific Resources) using a micropipette puller (Sutter Instrument), and fire polished on a microforge (Narashige Scientific Instrument Lab.) prior to use. Membrane currents were filtered at 5 kHz (−3dB) using a 4-pole low-pass Bessel filter digitized with a 12-bit A/D converter (DMA Interface, Axon Instruments), and stored for off-line analysis using personal microcomputers. Voltage paradigms were generated from a 12-bit D/A converter (DMA Interface, Axon Instruments) using pClamp software (Axon Instruments). After establishment of the whole-cell configuration, series resistance

and cell capacitance compensation were carried out prior to recording, and an 80% series resistance compensation was normally applied. No substraction of leakage current was made.

2.4. Solutions

The composition of the solutions used is shown in Table 1. The HBS solution was utilized for the bath perfusion. ATP (Sigma)-containing external solutions were prepared daily from 100 mM stock solution and was delivered from an U-tubing system as described previously (Erostegui et al., 1994). All the external solutions were

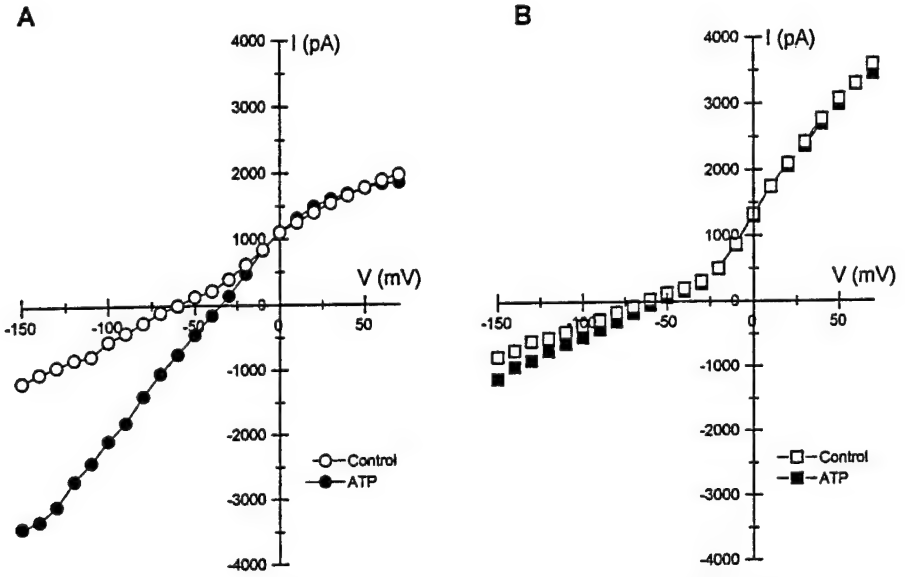

Fig. 1. Typical examples of ATP-induced effects on *I-V* relationships recorded from an OHC taken from a control (A) and noise-exposed animal (B). Currents were evoked by 60 ms steps from a holding potential of -60 mV. Normal HBS external solutions together with K^+-containing internal solutions were used. Currents were measured before (○, □) and during (●, ■) application of 100 μM ATP.

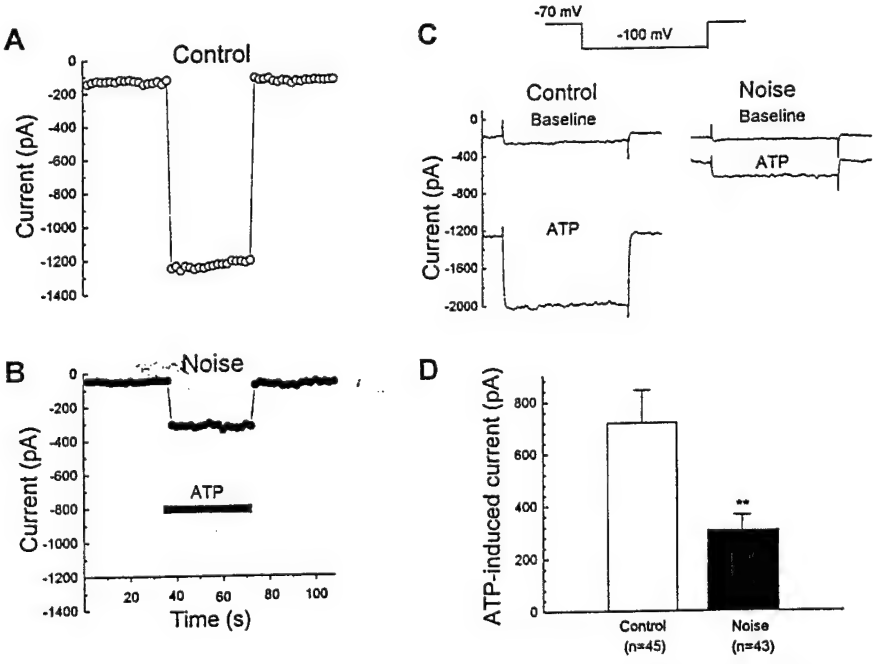

Fig. 2. A and B: typical examples of ATP (100 μM)-induced inward currents recorded from an OHC taken from a control (A) and noise-exposed animal (B). A holding potential of -70 mV and normal HBS external solution together with NMG^+-containing internal solution were used. C: superimposed examples of inward current traces recorded from an OHC taken from a control and a noise-exposed animal in the absence and presence of 100 μM ATP. Current was elicited by a 70 ms hyperpolarizing step to -100 mV from a holding potential of -70 mV. D: amplitudes (mean + SE) of ATP (100 μM)-induced inward currents obtained from OHCs taken from control and noise-exposed guinea pigs. Currents were measured at -100 mV using the step protocol in (C). Statistical significance was measured by ANOVA (** $P < 0.01$).

adjusted to a pH of 7.40 with NaOH and had a osmolality of 300 mosM. The internal solutions were adjusted to a pH of 7.35 with HCl and had a osmolality of 284 mosM. All experiments were conducted at room temperature (22 ~ 24°C).

Data are presented as means ± SE. Statistical significance was measured by chi-square test and analysis of variance (ANOVA), as appropriate. P values less than 0.05 were considered statistically significant.

Table 2
Incidence of response to ATP in OHCs from control and noise-exposed guinea pigs

Control		%	Noise-exposed		%
Response *	No Response *		Response *	No Response *	
25	22	53	14	62	18 **

* Number of cells are given.
** Statistical significance was measured by chi-square test ($P < 0.001$).

3. Results

Fig. 1 illustrates the current–voltage (I-V) relationships in the absence and presence of 100 μM ATP in OHCs from control and noise-exposed guinea pigs using normal K^+ internal and HBS external solutions. Currents were evoked by 60 ms steps from a holding potential of −60 mV. Monitored at −100 mV, the incidence of ATP responses was significantly reduced in cells from noise-exposed animals as compared to the incidence in cells from control animals (Table 2). In addition, the amplitude of the ATP induced inward current at −100 mV in cells from noise-exposed animals showed a tendency to be smaller than those recorded from control animals ($P = 0.06$).

To further examine this tendency, we isolated the ATP-induced currents by substituting N-methyl-glucamine (NMG^+) for K^+ in the pipette to block K^+ currents while using normal HBS external solution. Under such conditions, the incidence of the response to 100 μM ATP was not significantly different in the two groups of cells (noise: 88%, $n = 49$ vs. control: 98%, $n = 46$). However, the magnitude of 100 μM ATP-induced inward currents in cells from noise-exposed animals was decreased both at the holding potential of −70 mV (Fig. 2A, control; Fig. 2B, noise) and at the step to −100 mV (Fig. 2C control and noise). Examination of the data at −100 mV indicated that the effect was significant (noise: 303 ± 64 pA, $n = 43$ vs. control: 720 ± 125 pA, $n = 45$; $P < 0.01$; the zero change in response to ATP of the non-responders were not included; Fig. 2D).

To avoid the deleterious effects of increased intracellular Ca^{2+} caused by ATP and to further block outward K^+ current, we studied the effect of utilizing Ba^{2+}-containing external solution while keeping the same NMG^+-containing solution in the pipette. Some of the cells (n = 26, noise; $n = 27$, control) were the same ones studied in the previous paragraph. Results were similar to that using

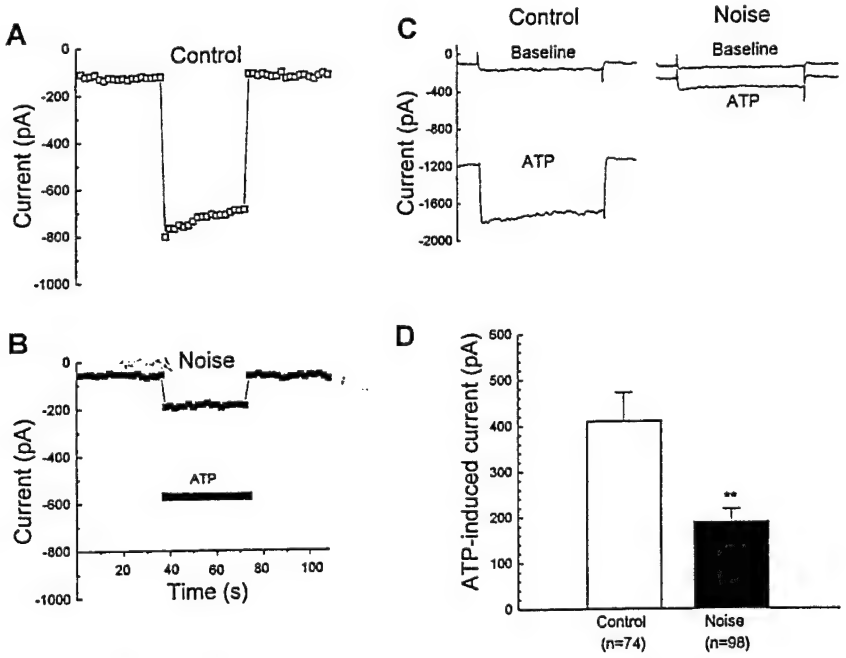

Fig. 3. ATP-evoked current response obtained in external Ba^{2+} solution. A and B: typical examples of ATP (100 μM)-induced inward currents recorded from an OHC taken from a control (A) and noise-exposed animal (B). A holding potential of -70 mV and 20 mM Ba^{2+}-containing external solution together with NMG^+-containing internal solution were used. C: superimposed examples of inward current traces recorded from an OHC taken from a control and noise-exposed animal in the absence and presence of 100 μM ATP. Current was elicited by a 70 ms hyperpolarizing step to -100 mV from a holding potential of -70 mV. D: amplitudes (mean + SE) of ATP-induced inward currents obtained from OHCs taken from control and noise-exposed guinea pigs. Currents were measured at -100 mV using the step protocol in C. Statistical significance was measured by ANOVA (** $P < 0.01$).

HBS external solution. There was no difference between groups in the incidence of the response (noise: 84%, $n = 117$ vs. control: 81%, $n = 91$). However, the magnitude of the ATP-induced currents in cells from noise-ex-

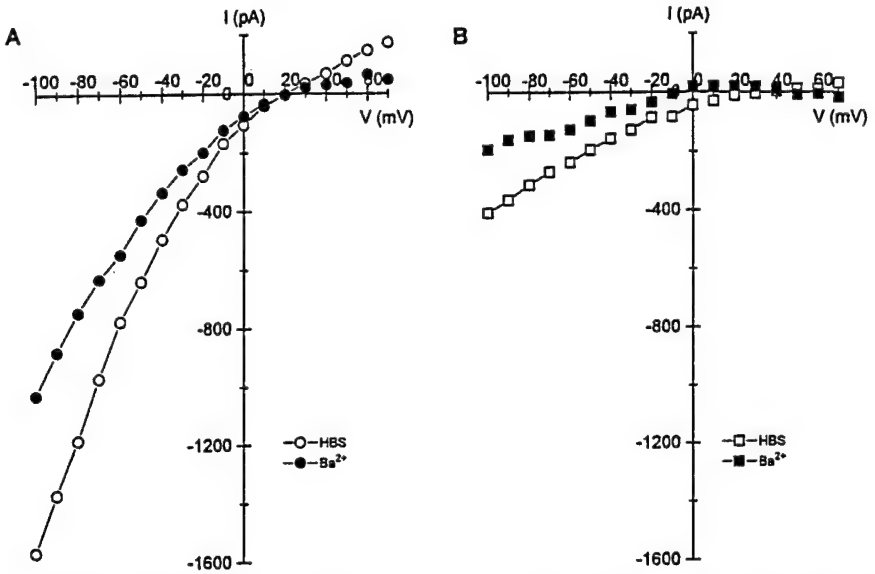

Fig. 4. ATP-evoked current at different voltages. Typical examples of 100 μM ATP-induced effects on *I-V* relationships recorded from an OHC taken from a control (A) and noise-exposed (B) animal. Data shown were obtained utilizing normal HBS external solutions (○), 20 mM Ba^{2+}-containing external solutions (●) and pipettes containing a NMG^{+}-containing internal solution. Plotted are the differences between currents in the presence and absence of the drug at the various test potentials. Currents were evoked by 70 ms steps from a holding potential of -70 mV.

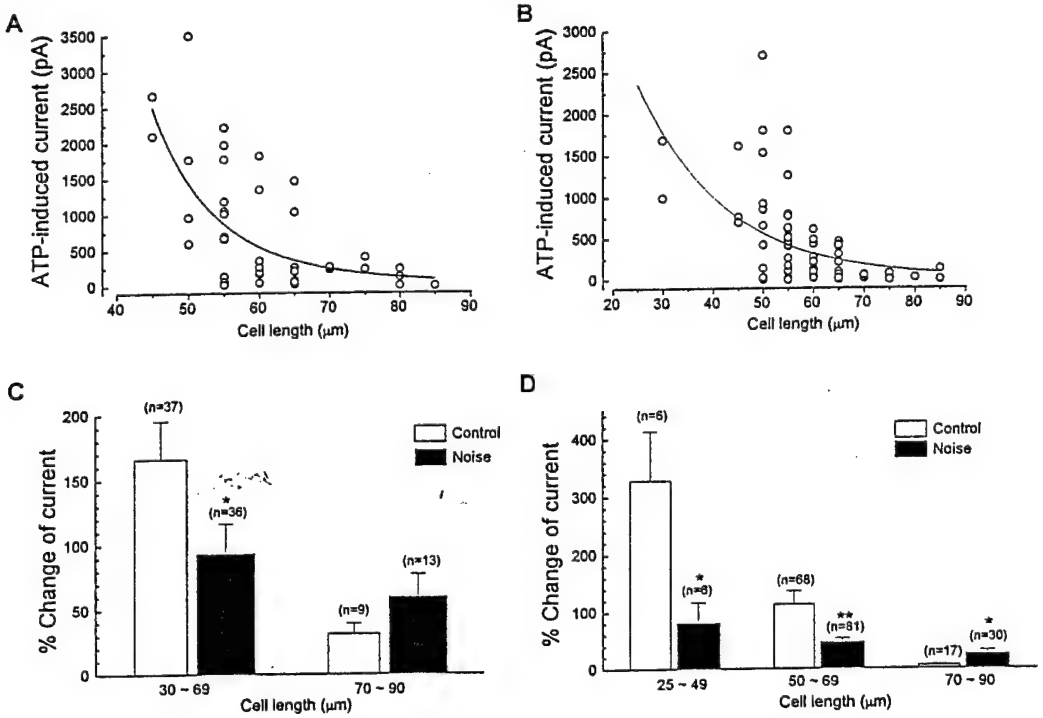

Fig. 5. A: scattergram of the 100 μM ATP-induced currents recorded from OHCs taken from control animals plotted against cell length. Data obtained utilizing normal HBS external solutions together with NMG^{+}-containing internal solutions. B: same as in (A) only data obtained utilizing 20 mM Ba^{2+}-containing external solutions together with NMG^{+}-containing internal solutions. C: comparison of the magnitude of responses to 100 μM ATP between OHCs taken from control and noise-exposed guinea pigs. Data obtained utilizing normal HBS external solutions together with NMG^{+}-containing internal solutions. D: same as in (C) only utilizing 20 mM Ba^{2+}-containing external solutions together with NMG^{+}-containing internal solutions. The divisions according to length were arbitrarily made. In some cases the same cell contributed data that appears in both normal and 20 mM Ba^{2+}-containing external solution groups. Each bar is mean + SE. Statistical significance was measured by ANOVA (* $P < 0.05$; ** $P < 0.01$). All current amplitudes were measured at -100 mV using a step protocol consisting of 70 ms hyperpolarizing steps to -100 mV from a holding potential of -70 mV.

posed animals was decreased both at the holding potential of −70 mV (Fig. 3A, control; Fig. 3B, noise) and at the step to −100 mV (Fig. 3C, control and noise). Examination of the data at −100 mV indicated that the response was significantly different from control (noise: 188 ± 29 pA, $n = 98$ vs. control: 410 ± 62 pA, $n = 74$; $P < 0.01$; Fig. 3D). Fig. 4 shows the *I-V* relationships of ATP-induced inward currents in OHCs from control (Fig. 4A) and noise-exposed (Fig. 4B) animals in HBS and Ba^{2+}-containing external solutions. The amplitudes of the ATP-elicited inward currents were smaller in Ba^{2+}-containing external solution than those in HBS solution. The pattern of results in OHCs from control and noise-exposed animals were similar.

Length of OHCs varies according to their position in the cochlear partition and is related to the function of OHCs (Pujol et al., 1992). We observed a length dependency for the ATP-induced effects in HBS (Fig. 5A) and Ba^{2+}-containing (Fig. 5B) external solutions for OHCs taken from control animals. This result is similar to that reported for acetylcholine-induced effects in OHCs (Erostegui et al., 1994). Fig. 5C (in HBS solution) and Fig. 5D (in Ba^{2+}-containing solution) illustrate the amplitudes of ATP-induced inward currents expressed as a percent of baseline currents (at −100 mV) in cells grouped according to length. The amplitudes of the currents were significantly decreased in the short cells (cell length < 65 μm) from noise-exposed animals as compared to those from controls. In contrast, ATP-induced currents were enhanced in cells with lengths over 65 μm from noise-exposed animals as compared to corresponding cells from controls.

4. Discussion

Results show that there were significant differences in the incidence and magnitudes of cation currents evoked by ATP (100 μM) in OHCs taken from guinea pigs exposed to chronic low-level noise when compared to the currents evoked from cells obtained from control animals. The response to ATP was decreased in short OHCs whereas in longer OHCs the response was increased.

At present we can only speculate as to the structural or biochemical mechanisms underlying our observations. The results are in harmony with the hypothesis that endogenously released ATP may be involved in the effects induced in the cochlea by low-level, chronic noise exposure. In other systems, the chronic release of ATP has been suggested to result in a down-regulation of purinoceptors (Maynard et al., 1992). This mechanism was proposed to explain the altered response to an ATP analogue in the rabbit isolated central ear artery after chronic electrical stimulation of the great auricular nerve (Maynard et al., 1992). By analogy, during noise exposure there may be a continuous exposure of OHCs to a high level of endogenous ATP which induces a similar down-regulation of ATP receptors in short OHCs. An up-regulation of ATP receptors may occur in long OHCs. Only future experiments can determine if such a mechanism accounts for the observed altered responses to ATP.

Alternatively, any noise-induced structural damage may have altered the response to ATP. We did not determine whether damage had occurred in the cochlear tissue we sampled. On the other hand, based on the literature to date, the noise level was probably not sufficiently intense to produce physical damage. No reports were found using the same band and level of noise in guinea pigs. However, the noise is the same level as one of the octave band of noise (0.5 kHz) exposures used by Bohne (1976). Bohne showed no damage to chinchilla hair cells at the light microscopic level after 9 days of continuous exposure to the 65 dB SPL noise. Others have shown that the guinea pig is slightly less susceptible to noise damage than the chinchilla (Decory et al., 1992). In other studies utilizing chinchillas, a more intense chronic noise exposure (85 dB SPL) resulted in stereocilia damage (Boettcher et al., 1992; Subramaniam et al., 1994). ATP receptors have been proposed to be located near the stereocilia on the apical surface membrane (Housley et al., 1992; Mockett et al., 1994). Thus the decreased response to ATP may be due to some physical or chemical alteration in the ATP receptors located near or on damaged stereocilia. On the other hand, the increased response observed in long OHCs argues against a physical damage and suggests a more complex mechanism.

Aside from damage, a subject's acute and chronic history of sound exposure changes the response of the cochlea to sound. Acute, low-level sound alters the mechanics of the cochlear partition in a complex manner as monitored by quadratic DPOAEs (Kujawa et al., 1995). Chronic, higher level exposure decreases the effect of subsequent noise exposure (Clark et al., 1987; Sinex et al., 1987; Canlon et al., 1988,1992; Campo et al., 1991; Franklin et al., 1991; Boettcher et al., 1992; Mensh et al., 1993; Subramaniam et al., 1994). During this latter effect which is called 'toughening', changes in the mechanics of the cochlea as monitored with DPOAEs have been reported (Franklin et al., 1991; Mensh et al., 1993; Subramaniam et al., 1994). In both these acute and chronic phenomena, the changes in DPOAEs and cochlear mechanics may indicate alterations in the function of the OHCs, possibly involving ATP. Whether the exposure utilized in the present study alters DPOAEs remains to be determined.

In summary, results indicate that the response to ATP was altered in OHCs obtained from noise exposed guinea pigs. We speculate that this is due to an alteration in the number of ATP receptor proteins. Additional research will be necessary to determine whether this mechanism actually underlies the observed phenomena.

Acknowledgements

Thanks to Maureen Fallon, Ruth Skellett, and Christopher LeBlanc, for their technical help and to Sharon Kujawa for advice. Supported by DAMD 17-93-V-3013, NIH grant R01-DC00722, Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation.

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APPENDIX #7

DAMD17-93-V-3013

Bobbin and Berlin

2aPP30. A suppressive "off-effect" in the f_2-f_1 DPOAE response to continuous, moderate-level primary stimulation. Sharon G. Kujawa, Maureen Fallon, and Richard P. Bobbin (Kresge Hearing Res. Lab., LSU Med. Ctr., 2020 Gravier St., Ste. A, New Orleans, LA 70112)

Following a short period of amplitude enhancement, the f_2-f_1 DPOAE can be suppressed during continuous ipsilateral stimulation with primary tones. Evidence for and against efferent control of such response alterations has been presented. Here, an exacerbation of f_2-f_1 suppression, apparent following a short rest from continuous stimulation, is described and the hypothesis that it is efferent-mediated is tested. The f_2-f_1 DPOAE to intermittent or continuous primaries ($f_1=6.25$ kHz; $f_2=7.5$ kHz; $L_1=L_2=60$ dB SPL) was recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles before, during, and after perfusion of the cochlear perilymph compartment with antagonists of olivocochlear efferent activity (curare, 1 μM; bicuculline, 10 μM) or of action potentials in general (tetrodotoxin, TTX; 1 μM). Results confirmed an initial f_2-f_1 amplitude enhancement and gradual suppression during continued stimulation. Following a 1-min rest, however, the DPOAE was further suppressed from its pre-rest level. Thereafter, its amplitude increased rapidly, reaching a maximum within 1–2 min. These amplitude alterations were intensity dependent. Moreover, they were reduced, but not blocked by efferent antagonists and were largely unaffected by TTX, suggesting that additional (e.g., local) alterations in cochlear status impact the cochlear mechanical response to continuous sound stimulation. [Work supported by NIH DC00007; DAMD 17-93-V-3013.]

Journal of the Acoustical Society America, 95, 2845, 1994.

APPENDIX 8

FIGURES FOR THE HUMAN STUDIES.

CHAPTER: PROTECTING THE AUDITORY SYSTEM AND PREVENTION OF HEARING PROBLEMS

PROJECT DIRECTORS: RICHARD P. BOBBIN, PH.D AND CHARLES I. BERLIN, PH.D.

Figure Legends:

Fig 1 Echo Lab showing Emission being collected relative to noise background.

Fig 2. Echo Lab showing analysis of 256 accepted sweeps and 7 rejected sweeps,

Fig 3. Constructing stimuli consisting of binaural clicks and binaural noise preceding the click by 2.0 msecs.

Fig 4. Verifying flat click spectrum from stimulus waveform.

Fig 5. Threshold data for 1500 Hz tone bursts; detection and amplitude of emission.

Fig 6. Data from 7 subjects confirming that 1500 Hz tonebursts behave like clicks in previous experiments.

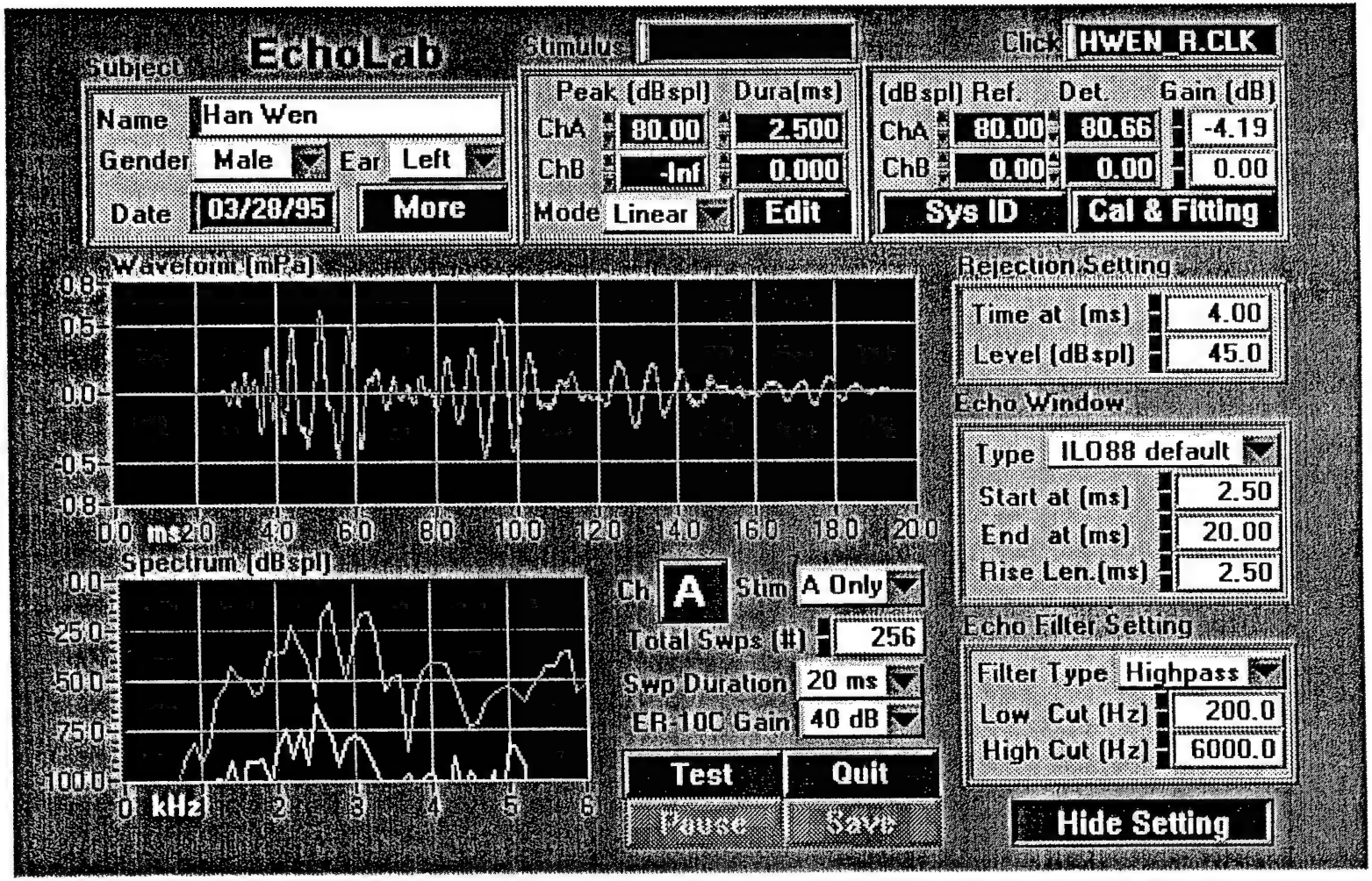
EchoLab
Subject
Name
Han Wen
Gender
Male
Ear
Left
Date
03/28/95
More
Stimulus
Click
HWEN_R.CLK
Peak (dBspl)
Dura(ms)
ChA
80.00
2.500
ChB
-Inf
0.000
Mode
Linear
Edit
(dBspl)
Ref.
Det.
Gain (dB)
ChA
80.00
80.66
-4.19
ChB
0.00
0.00
0.00
Sys ID
Cal & Fitting
Waveform (mPa)
0.8
0.5
0.0
-0.5
-0.8
0.0 ms
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
Spectrum (dBspl)
0.0
-25.0
-50.0
-75.0
-100.0
0 kHz
1
2
3
4
5
6
Ch
A
Stim
A Only
Total Swps (#)
256
Swp Duration
20 ms
ER-10C Gain
40 dB
Test
Quit
Pause
Save
Rejection Setting
Time at (ms)
4.00
Level (dBspl)
45.0
Echo Window
Type
IL088 default
Start at (ms)
2.50
End at (ms)
20.00
Rise Len.(ms)
2.50
Echo Filter Setting
Filter Type
Highpass
Low Cut (Hz)
200.0
High Cut (Hz)
6000.0
Hide Setting

Figure #1

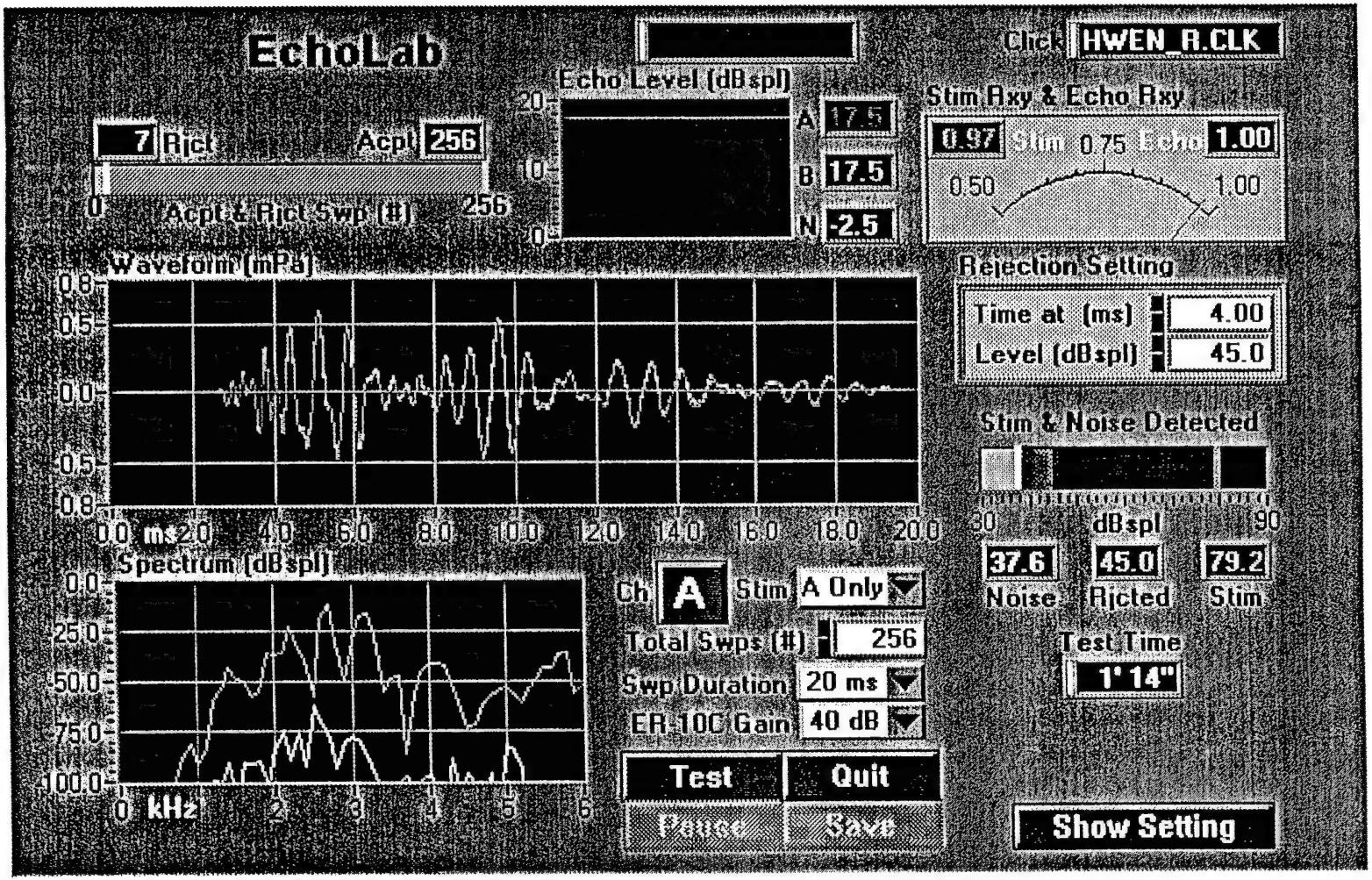
EchoLab
Click HWEN_R.CLK
Echo Level (dBspl)
20
10
0
A 17.5
B 17.5
N -2.5
Stim Rxy & Echo Rxy
0.97 Stim 0.75 Echo 1.00
0.50
1.00
7 Rjct
Acpt 256
0 Acpt & Rjct Swp (#) 256
Waveform (mPa)
0.8
0.5
0.0
-0.5
-0.8
0.0 ms 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0 20.0
Rejection Setting
Time at (ms) 4.00
Level (dBspl) 45.0
Stim & Noise Detected
30 dBspl 90
37.6 Noise
45.0 Rjcted
79.2 Stim
Test Time
1' 14"
Spectrum (dBspl)
0.0
25.0
50.0
75.0
100.0
0 kHz 2 3 4 5 6
Ch A
Stim A Only
Total Swps (#) 256
Swp Duration 20 ms
ER-10C Gain 40 dB
Test
Quit
Pause
Save
Show Setting

Figure #2

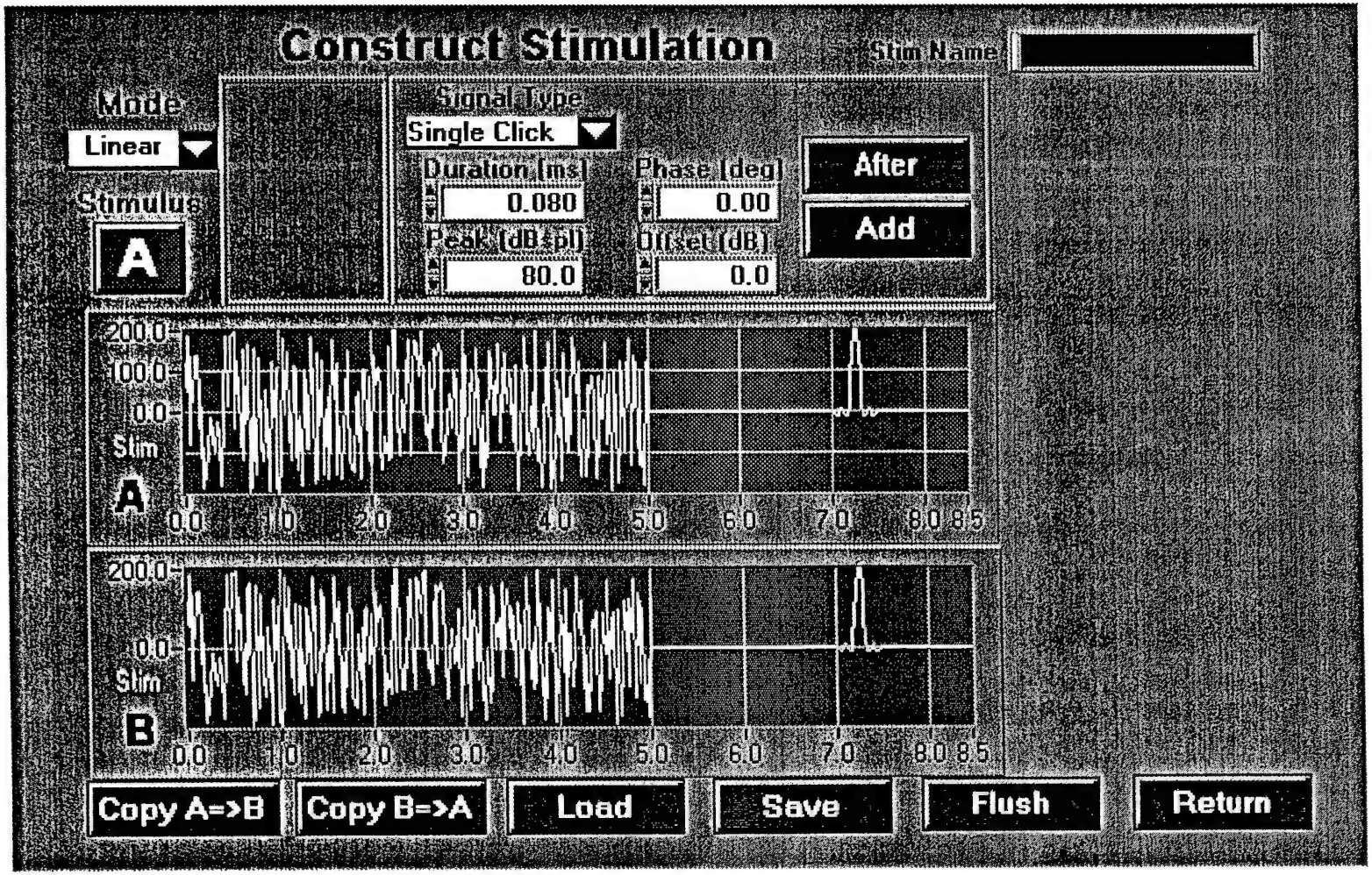
Construct Stimulation
Stim Name
Mode
Linear
Stimulus
A
Signal Type
Single Click
Duration [ms]
0.080
Phase [deg]
0.00
Peak [dB spl]
80.0
Offset [dB]
0.0
After
Add
200.0
100.0
0.0
Stim
A
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 8.5
200.0
0.0
Stim
B
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 8.5
Copy A=>B
Copy B=>A
Load
Save
Flush
Return

Figure #3

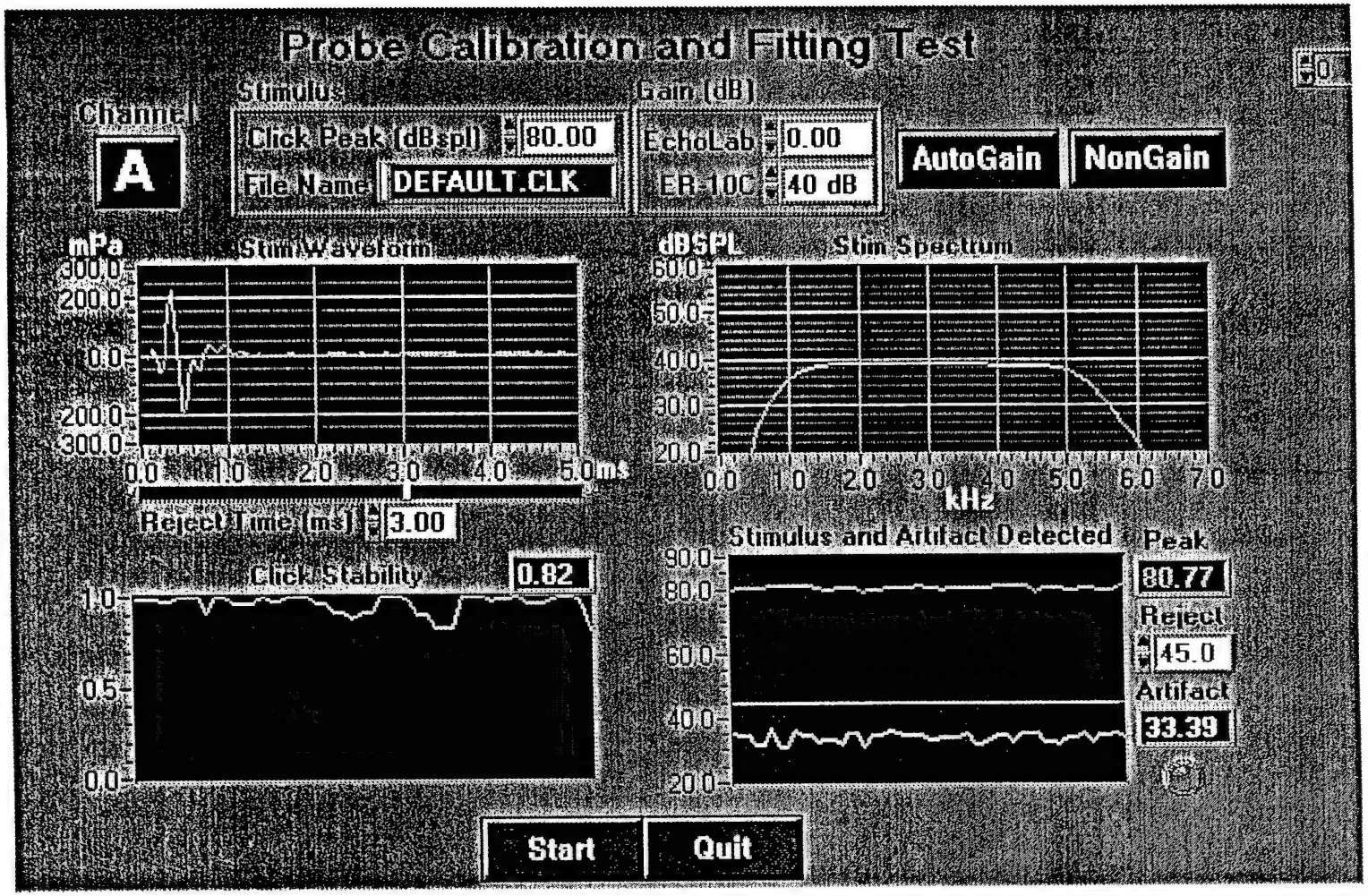
Probe Calibration and Fitting Test
Channel
A
Stimulus
Click Peak (dBspl) 80.00
File Name DEFAULT.CLK
Gain (dB)
EchoLab 0.00
ER-10C 40 dB
AutoGain
NonGain
mPa
Stim Waveform
300.0
200.0
0.0
200.0
300.0
0.0 1.0 2.0 3.0 4.0 5.0 ms
dBSPL
Stim Spectrum
60.0
50.0
40.0
30.0
20.0
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0
kHz
Reject Time (ms) 3.00
Click Stability 0.82
1.0
0.5
0.0
Stimulus and Artifact Detected
90.0
80.0
60.0
40.0
20.0
Peak
80.77
Reject
45.0
Artifact
33.39
Start
Quit

Figure #4

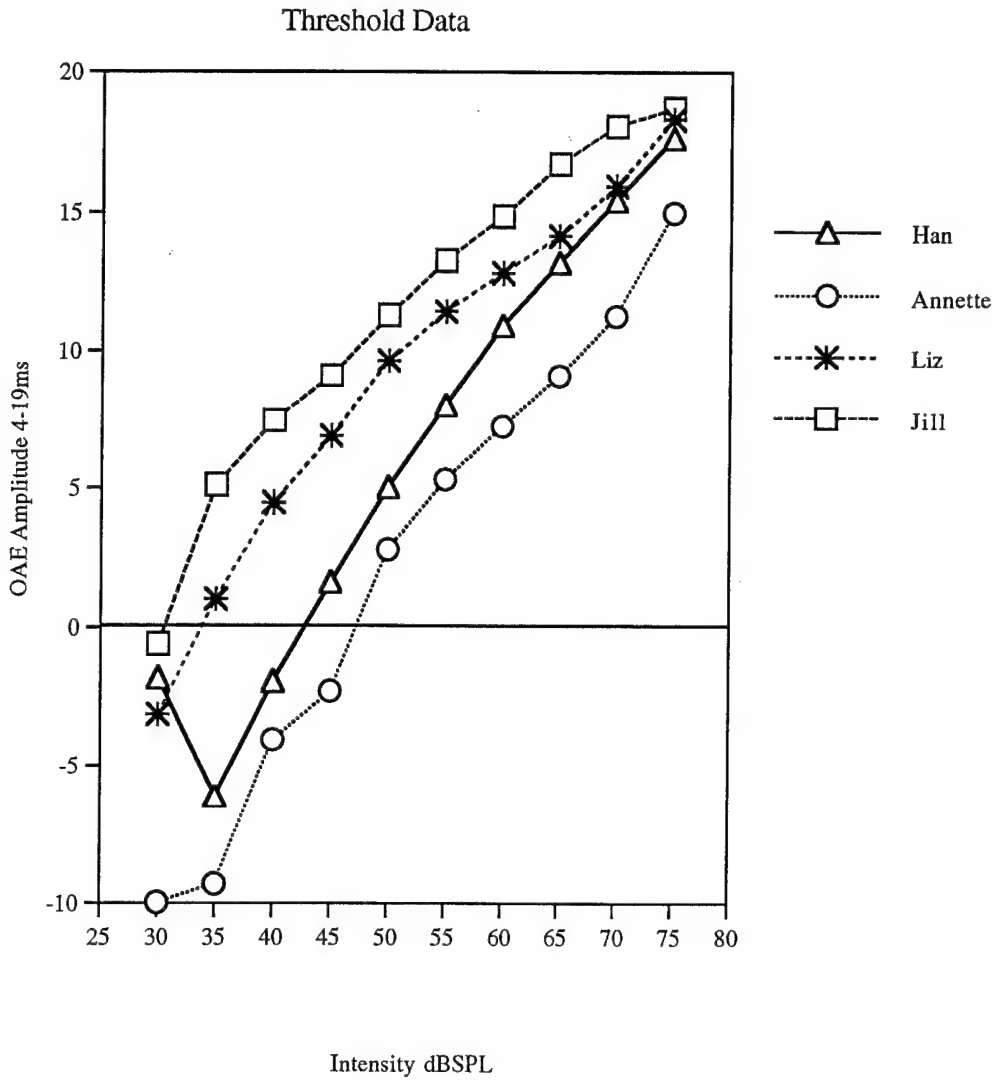
Threshold Data
20
15
10
5
0
-5
-10
OAE Amplitude 4-19ms
25
30
35
40
45
50
55
60
65
70
75
80
Intensity dBSPL
Han
Annette
Liz
Jill

Figure #5

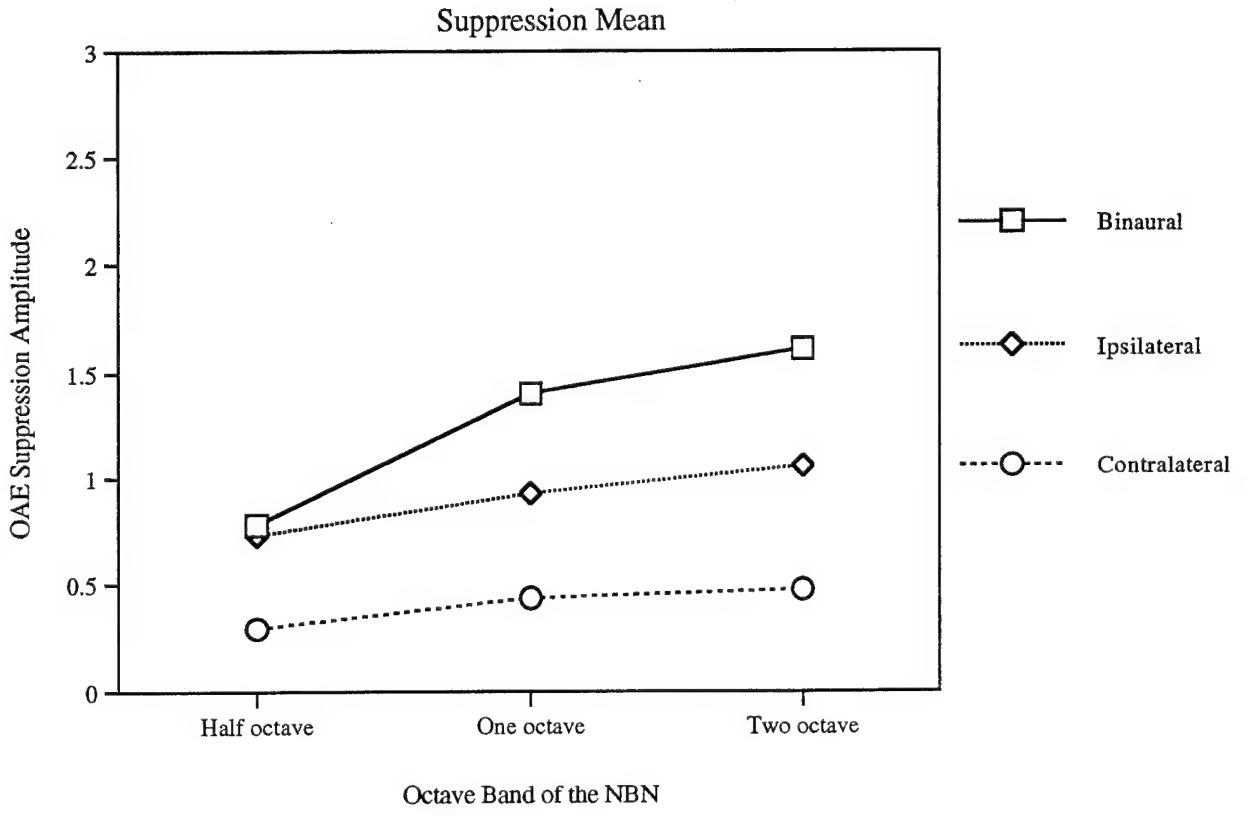
Suppression Mean
OAE Suppression Amplitude
3
2.5
2
1.5
1
0.5
0
Half octave
One octave
Two octave
Octave Band of the NBN
Binaural
Ipsilateral
Contralateral

Figure #6

APPENDIX #9

DAMD17-93-V-3013

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ELSEVIER

Hearing Research 2390 (1995) xxx C

HEARING RESEARCH

Binaural noise suppresses linear click-evoked otoacoustic emissions more than ipsilateral or contralateral noise

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Received 7 November 1994; revised 25 March 1995; accepted 4 April 1995

Abstract

We studied the efferent suppression of click-evoked otoacoustic emissions with 65 dB SPL of white noise presented to left, right, or sometimes both, ears for 408 ms. Each burst of noise preceded a series of four unipolar 80 usec 65 dB peak Sound Pressure clicks, presented to the left ear only. The first click of the four-click group followed the end of the noise by either 1, 2, 5, 10, 20, 50, 100 or 200 ms; each subsequent click was offset by 20 additional ms via an ILO88 system with special programming modifications. Conditions were alternated so that a 'without noise' condition preceded a 'with noise' condition for three repetitions of 600 clicks per trial. Seven subjects with normal hearing participated in the study, and three of the seven participated in a test-retest reliability study. Results showed the greatest suppression followed binaural stimulation ending within one to five ms of the first click in the pulse train. Somewhat less suppression was seen following ipsilateral stimulation. The least amount of suppression was seen following contralateral stimulation, suggesting that previous research using contralateral stimulation may underestimate efferent effects. We saw no effects when the end of the noise was 100 ms or more away from the beginning of the click train.

Keywords: Otoacoustic emissions; Efferent suppression; Binaural; Ipsilateral; Contralateral; Forward masking

1. Introduction

The medial olivocochlear system suppresses segments of outer hair cell activity when activated either contralaterally, ipsilaterally or bilaterally with an auditory stimulus of sufficient duration (Warr et al., 1986; Warr and Guinan, 1978; Puel and Rebillard, 1990; Liberman, 1989; Kujawa et al., 1993,1994).

Previous experiments on suppressing otoacoustic emissions in humans have focused mostly on the suppressive effects of continuous contralateral stimulation (e.g., Collet et al., 1990; Ryan et al., 1991; Berlin et al., 1993a,b; Berlin et al., 1994).

Kevanishvili et al. (1992) and Gobsch et al. (1992) studied forward masking of emissions in an attempt to relate the detection of hair cell suppression to perceptual masking. They used either unipolar clicks or 1000 Hz tone bursts, with time separations of 5 to 200 ms and masker durations of 6 and 50 ms; they saw little ipsilateral suppressive effect of the low level maskers on emission amplitude when judged subjectively by three independent judges.

Henson (1993) offered a comprehensive summary of efferent contralateral effects on otoacoustic emissions. He noted that most of the published work showed suppression or reduction of emission during contralateral acoustic or electrical stimulation; however, some workers, notably Brown and Norton (1990), and Plinkert and Lennarz (1992), reported an occasional increase (as well as decrease) in emission amplitude as a result of contralateral stimulation.

In this work we presented unipolar clicks to seven normal hearing human subjects to show the suppressive effects of low level binaural, ipsilateral, contralateral white noise (408 ms in duration) in a forward masking paradigm. We used a proprietary analysis system (Wen et al., 1993) to record differences of as much as 7 dB between control and experimental traces that were not immediately apparent to casual observation.

2. Methods

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SSDI 0378-5955(95)00082-8

The ILO88 system is currently widely used for the recording of transient evoked emissions (Kemp et al., 1989). In the default so-called non-linear condition three 80 dB peak sound pressure pulses are presented in one polarity while a fourth pulse is presented out of phase with the first three and at 10 dB greater intensity. The resulting echo represents the difference between the sum of the three echoes elicited at 80 dB and the one out-of-phase echo elicited by the 90 dB pulse; the strategy is designed to cancel any artifactual ringing which could be mistaken for hair cell echoes.

Suppression is evident, but not robust, with these default conditions (Berlin et al., 1993b). In contrast, efferent effects are more readily seen at low intensities than at high and with linear rather than non-linear click trains (Collet et al., 1990; Hood et al., 1994); therefore, we designed our experiments around low level linear clicks and noises.

One of us (D.K.) supplied the program for an ILO88 system to control the temporal interval between the offset of a duration-controlled noise stimulus and the onset of a 4-click train. We selected a 408 ms duration noise based on the work of Liberman (1989) and Huang et al. (1994) to maximize the likelihood of activating the efferent system. The emission-evoking 80 usec clicks were all 65 db peak sound pressure and all of the same polarity. The first click of the four-click group followed the end of the noise by either 1, 2, 5, 10, 20, 50, 100, or 200 ms, whereas the final three clicks followed the first click by successive increments of 20 ms. Conditions were alternated so that a 'without noise' condition preceded a 'with noise' condition for three complete trials. The order of ipsilateral vs. contralateral vs. bilateral presentation of the noise was counterbalanced among subjects for the 24 different listening conditions. Three subjects were retested under conditions where the first of the four clicks began 1 ms after the termination of the noise and each subsequent click was offset by 20 ms.

Each set of control vs. experimental data consisted of the mean of three 'without noise' trials, and three 'with noise' trials, compared to one another using two separate quantification systems. In one case we compared the mean echoes to each other using the Kemp aggregate echo level number, which we called 'dB ILO'. This number represents the overall spectral amplitude of the echoes averaged by the ILO88 system over a 20.48 ms window. The second method used the Kresge Echomaster system (Wen et al., 1993) which allows custom designed amplitude, time and frequency comparisons between means of control and experimental conditions. We chose to quantify the RMS differences between the echoes in two ms segments and labeled this number 'dB-K' to differentiate it from the overall RMS number available from the ILO88 system. The Kresge Echomaster system also allowed us to make temporal comparison of differences between segments of the control and experimental echoes in 40 usec steps (See Table 1 later for examples).

3. Results

The binaural noise condition generated 1.5 to 2 dB-ILO of emission suppression when the noise preceded the first click in the train by one to twenty ms. Thereafter the suppressive effects decreased as time-separation increased. The amount of suppression to binaural noise shown in Fig. 1 is 2.5 to 4 db-K between 8 and 18 ms after the click

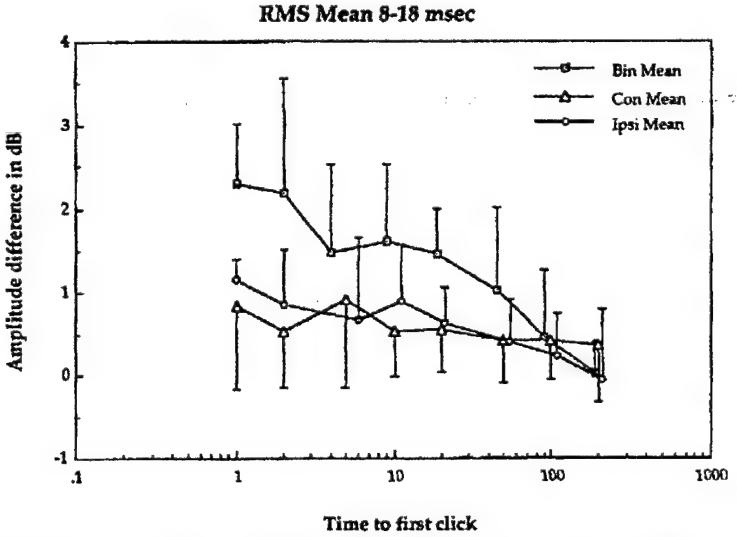

Fig. 1. The effects of 408 msecs of binaural, contralateral, or ipsilateral noise preceding a left ear click train in which the first click started at 1, 2, 5, 10, 50, 100, and 200 msecs after the end of the noise burst. Each of the subsequent clicks was offset by an additional 20 msecs. The mean and 1 Standard Deviation are presented using the amplitude difference data between 8–18 msecs from the Kresge Echomaster (KEM) 3.0 program.

stimulation, a zone in which the majority of suppression is seen. The values for ipsilateral and contralateral suppression are respectively smaller in this forward masking paradigm. Fig. 2 compares suppressive effects of binaural, ipsilateral and contralateral noise at 1, 10, 50 and 200 ms time separations between the end of the noise and start of the first click. The suppression is expressed in db-K.

Fig. 3 offers another view of the same data. Here we compare the data in db-K over the four to 20 ms period of the KEM 30 program analysis, showing the binaural data only when the noise terminates 1, 5, 20, and 200 ms before the first click. Then in the companion figure we show similar binaural data for time-separations of 2, 10, 50 and 100 ms between the end of the noise and the beginning of the first click. Again three primary trends are evident:

1. The shorter the time-separation between the end of the noise and first click, the greater the suppressive effect ($F = 6.44$; df = 7,42; $P < 0.001$). Duncan's Range Test showed that 1 ms time separation is most effective. There were no significant differences between 2, 5, 10, and 20 ms of separation. Thereafter separations of 50, 100, and 200 ms all differed significantly from one another.
2. Suppression of 2.5 to 3.5 dB-K takes place in the eight to eighteen ms zone after click onset ($F = 10.37$; df = 8,48; $P < 0.001$). Duncan's range Test showed that the greatest effects occur between 18 and 20 ms. No significant differences were seen between 10 and 18 ms, while the least suppression was seen in the 2 to 8 ms ranges.
3. Binaural noise generates more efferent suppression than either ipsilateral or contralateral noise ($F = 11.43$; df = 2,12; $P < 0.005$). Duncan's Range Test showed that, while binaural noise generated the most suppression, ipsilateral and contralateral stimulation did not differ from one another in their suppressive abilities, although they did exert significant amounts of suppression. We also observed that the sum of ipsilateral and contralateral suppression was within 0.8 of a dB of the suppression generated by binaural noise as was predicted by Kirk and Johnstone (1993).

Spectral analysis through a Hanning window available in the KEM 30 program showed a gradual shift in the largest spectral difference (at 2344 Hz) from 6.328 dB of binaural-noise-induced-suppression when the first click followed the noise by only one ms, dropping to 2.267 dB at 100 ms time separation, and then down to 0.394 dB at 200 ms time separation for one representative subject (CB). Other subjects showed qualitatively similar suppression in spectral zones between 1000 and 3000 Hz.

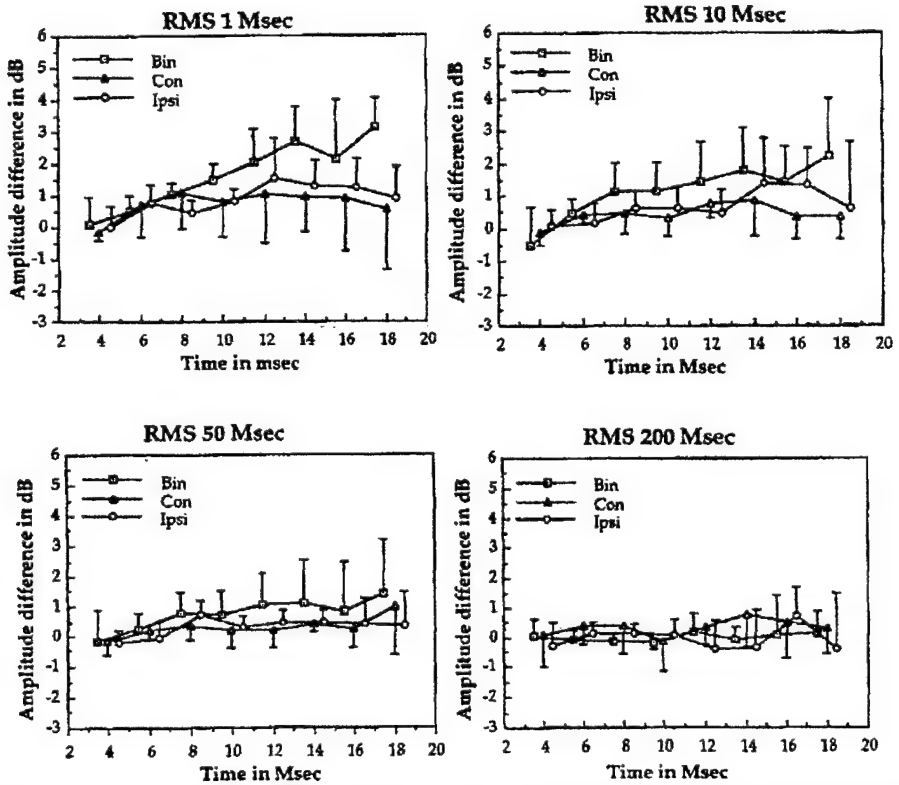

Fig. 2. The emission-suppressing effects of binaural, ipsilateral and contralateral noise. Data, expressed in db-K, are grouped by time intervals in which the noise preceded the first of four click-evoked emission by either 1, 10, 50 or 200 msecs.

We checked test-retest reliability for three subjects. Data for one subject are shown in Fig. 4. Group analysis shows reliability as high as 0.62 for the binaural data at 1 ms time-separation but only 0.13 for the ipsilateral data and.046 for the contralateral data. Reliability in general fell as the time separation between the end of the noise and the onset of the click increased. This observation suggests that the binaural effects are clearly more robust and more reliable than either ipsilateral or contralateral stimulation in this forward masking paradigm with humans.

4. Discussion

There are two major efferent systems which can affect the emissions reaching the recording microphone: the middle ear muscle reflex efferent motor loop, and the olivocochlear efferents. The 80 usec click stimuli in this experiment were just detectable by our normal subjects at 38 dB peak sound pressure. Thus, since the clicks used in the experiment were presented at 65 dB peak sound pressure, they were only at 27 dB HL, a level far too faint to evoke a clinically measurable middle ear muscle reflex. The threshold for the noise was 22 dB SPL; therefore, the 65 dB SPL noise was only 43 dB HL, also a level far too low to elicit a clinical middle ear muscle reflex.

The suppressive effects are actually largest when the clicks and the noise are at lower intensities. We know this from separate work completed after this present experiment had started, which showed us that the suppressive effects of contralateral noise were largest when the clicks were at 55 dB peak sound pressure; the relative effects diminished when either the noise or the clicks were presented at higher intensities (Hood et al., 1994), a phenomenon also reported by Collet et al. (1990).

Finally, studies of people with no middle ear muscle function (e.g., Collet et al., 1990; Berlin et al., 1993a) all suggest that the middle ear muscle reflex did not participate in this experiment, although of course it is impossible to completely rule out subclinical middle ear muscle contraction in any one subject.

Still another potential confounding problem in such

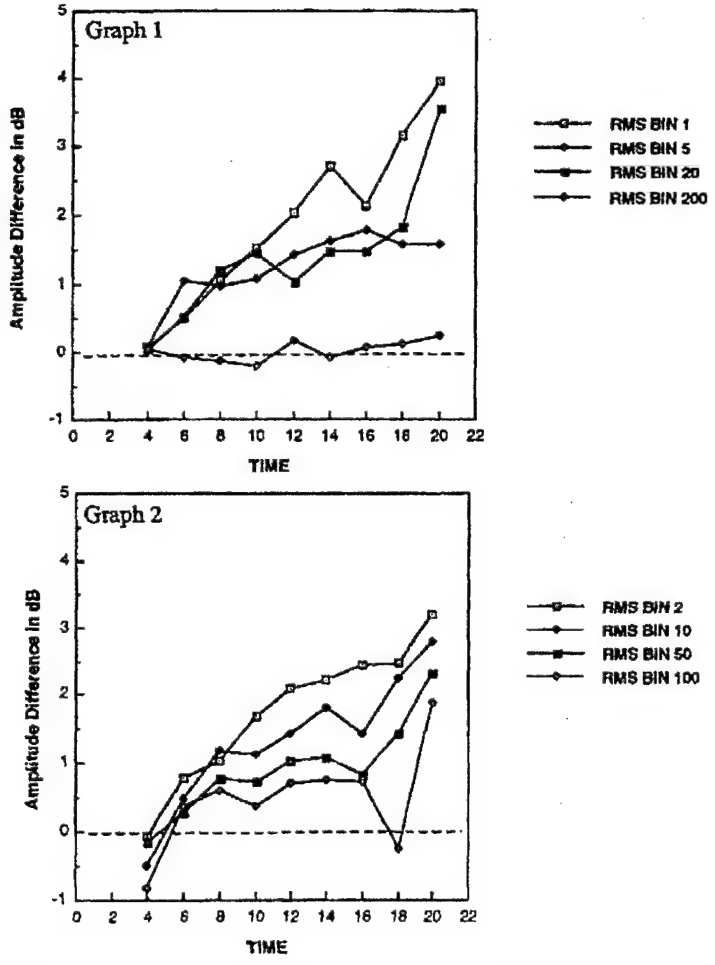

Fig. 3. Shows binaural noise suppression data for 1, 5, 20 and 200 msecs of time-separation between the end of the noise and the beginning of the first of four clicks. The companion figure shows the data for 2, 10, 50 and 100 msecs. Data are expressed in db-K.

experiments is acoustic crosstalk. If standard ear phones such as TDH-39 with MX 41-AR cushions had been used, crossover by bone conduction or even partly by air conduction could conceivably take place at levels as low as 40 dB Hearing Level. However, in this experiment we used insert earpieces from the Kemp system; the psychophysical crossover in similar insert earphones exceeds 70–90 dB in frequencies below 1000 Hz and 60–70 dB in frequencies above 1000 Hz (Killion, 1984).

Liberman and Brown (1986) showed almost no efferent suppression in cats stimulated by 25 ms or less of noise stimulation; optimum durations to activate the efferents were reported to reach an asymptote between 50 and 500 ms. Within the limits of the technical difficulties to be described, our findings suggest that the human efferent system overlaps at least one part of the time-frame seen in cats.

Binaural stimulation in the forward masking paradigm predictably elicited more robust and more reliable efferent suppression of evoked otoacoustic emissions than either ipsilateral or contralateral stimulation. In absolute numerical terms, however, more suppression is seen with continuous 60 dB SPL contralateral noise stimulation when the click is at 55 dB peak Sound Pressure (approx. 17 dB HL) than we see in the binaural condition in this forward masking experiment (Hood et al., 1994; Berlin et al., 1994). This observation is to be expected because of the forward masking nature of the paradigm; the continuously running noise paradigm would confound data collection during conditions of ipsilateral and binaural efferent stimulation.

5. Technical difficulties

We recognize several problems with the data presentation in this experiment. Because of constraints in the available software, the click stimuli could only be delivered in packets of four stimuli per stimulation unit. Thus, when we described a click train as beginning 'one ms after the end of the noise,' it was only the first of the four clicks that was one ms away from the end of the noise. The other

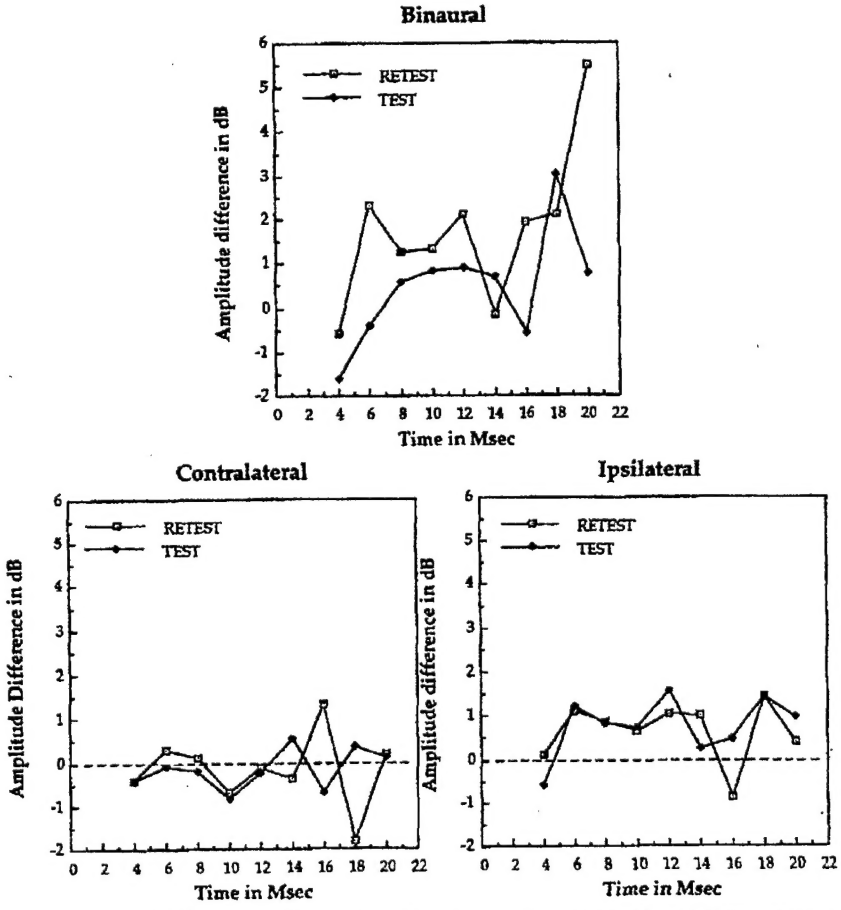

Fig. 4. One subject's test-retest reliability for the time separation of 1 ms between the end of the binaural, ipsilateral or contralateral noise.

three pulses were 21, 41, and 61 ms away from the end of the noise respectively. Yet each of the responses to the clicks is added into the average obtained by the ILO88. Similarly, in the fifty ms condition the last three clicks were presented 71, 91, and 111 ms after the end of the noise. Thus, whatever efferent effects we report here are likely to have been attenuated because three-quarters of the 600 clicks used to comprise a single file were 20 to 60 ms later than the intended time-relationship to the end of the noise.

We are indebted to G.I. Frolenkov (Frolenkov et al., 1995 and Tavartkiladze et al., 1995) for pointing out to us that, in order to avoid acoustic interaction between the end of the noise and the beginning of the first click, we should have allowed the noise about 5 ms to decay. This was not a fatal flaw in any of our data collection since only the first click in the series was within 1–5 ms of the offset of the noise; all subsequent clicks were offset by at least 20 ms.

If we were to present only a single click after the end of the noise, the 80 ms window averaging paradigm used by the ILO88 would still include the data from the three subsequent empty 'vacated' bins as 'noise' and would attenuate the apparent size of the averaged evoked emissions by a factor of three. [We have recently observed that a one-click experiment in this paradigm yields about the same data as a four click train experiment; in addition we find that there may be gender differences, laterality effects and occasionally even reverse suppression effects which have to be taken into consideration in future experiments (Berlin et al., 1995; Barham et al., 1995)].

The broad-band click and broad-band noises potentially ignore frequency specificity reported in experiments of this sort (e.g., Liberman et al., 1989). Thus an improved experiment would have all of the echo-evoking stimuli in the same time registration with respect to the end of the noise, would include the echoes from only a single 20.48 ms bin following the click, would take into consideration ear and gender effects, and would focus on various frequency bands, where presumably the effects might be even larger than we report here.

6. OF what value is a three to six dB effect in the auditory system?

A forward masking effect of 3 to 6 dB K suppression of hair cell activity, which we saw when both ears were exposed to approximately a half-second of noise, would be even larger if it could be measured while the noise were continuously active. This work supports Liberman's prediction that 200 ms or more durations of noise would adapt outer hair cell function leading to a change in the excitation pattern of inner hair cells and single units. Liberman proposed that the presence of the noise probably changed the baseline operating characteristics of the outer hair cells and would adapt single units faster, in preparation for upcoming transients; if such a shift were to be applied to the sharply rising edge of a speech intelligibility curve such as the articulation index, (Pavlovic, 1994) it could

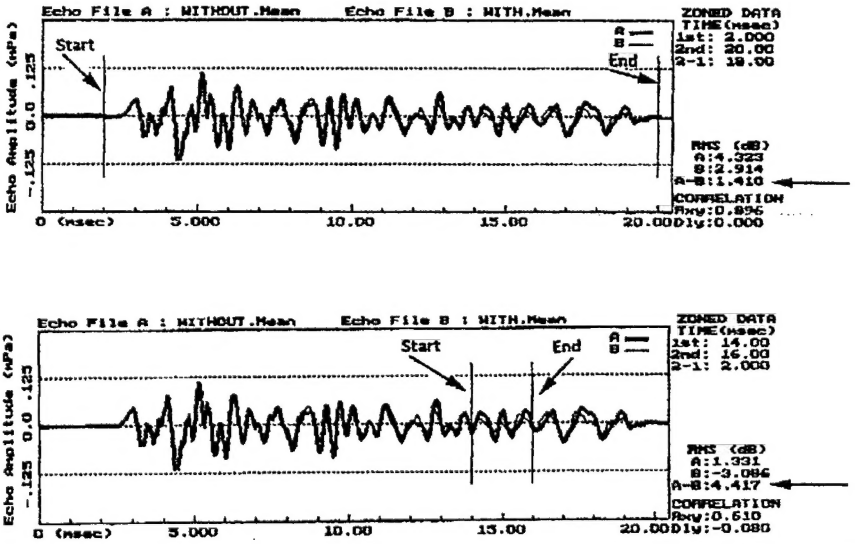

Fig. 5. Two averaged emissions traces (Echo File A = the mean of three trials without noise compared to Echo File B = the mean of three trials with noise) appear to have only 1.410 dB difference between them when scanned from 2 through 20 msecs (vertical bars marked **Start** and **End**) but, in fact, have 4.417 dB amplitude differences between them when scanned between 14 through 16 msecs after stimulation. See numerical codes to the right of each display for clarification. Companion Table 1 shows actual data values for these traces for Time Segment in msecs, Rxy (correlation), RMS differences, and Time Delay between traces in msecs.

conceivably improve connected speech intelligibility in a borderline noisy situation by as much as 40 to 60% (see for example Humes et al., 1986; Pavlovic, 1994; Hood et al., 1991). Thus, shifts in the baseline from which listening in noise takes place, or anti-maski9X phenomena as outlined by Hirsh (1948), Licklider (1948) Nieder and Nieder (1970), Kawase et al. (1993), or Kawase and Liberman (1993), might be traced to outer hair cell changes controlled by the efferent nervous system, which helps to facilitate listening in noise. We would not expect the effects of efferent function to be dramatic and outstanding in humans (cf. Scharf et al., 1994) without the conditions which mimic real-life listening conditions, including (preferably) binaural presence of long durations (200 ms or more) of noise. However, Henson et al. (1994) suggest that cochlear reverberation may be reduced through activation of the efferents in bats. A similar reduction in cochlear reverberation, as yet unreported in humans, would serve to essentially improve the signal-to-noise ratio whenever it occurred.

7. Relationship to other forward masking work on emissions

Kevanishvili et al. (1992), and Gobsch et al. (1992) presented work studying the relationship of forward masking to emission suppression. They studied whether perceptual masking occurred at the hair cell and cochlear partition level, or at more central levels. They assumed that if masking were taking place at the pre-neural level, perceptual masking and masking of the emissions would occur at the same intensities and durations. They reported that the perceptual masking functions and masking of the emissions were quite separate, and in fact used maskers of 80 and 68 dB SPL to prove that point. Whether or not they induced middle ear muscle reflexes is debatable, but the 5 ms delay between noise offset and click onset should have vitiated most if not all of those effects. Yet these conscientious workers did not report that they saw much masking of the emissions. Our contention, after looking at their waveforms, is that there are many amplitude and phase changes in the tracings that they published, which could be easily ignored by someone looking simply at patterns but that could be quantified by summing traces from similar conditions and overlapping conditions with and without masking. Any deviations from perfect correlation might be due to either noise or phase and amplitude changes. Our analysis program is capable of quantifying such a change, where the eye is not. For example, using data that do not appear to show much difference between traces by casual inspection, we can show differences of 4.417 dB (Fig. 5) between 14 and 16 ms but only 1.4 dB when viewed globally over a 2–20 ms period after stimulation. Such minuscule visual differences in Grobsch et al. (1992) and Kevanishvili et al. (1992), could easily be overlooked by

Table 1
Echo RXY, RMS differences and delay values for Fig. 5

Time (ms)	Rxy (x = A, y = B)	RMS (A–B in dB)	Delay (B to A in ms)
2.00–4.00	0.972	0.14	0.000
4.00–6.00	0.964	0.35	0.000
6.00–8.00	0.950	0.99	0.000
8.00–10.00	0.867	1.96	−0.040
10.00–12.00	0.913	2.36	−0.040
12.00–14.00	0.836	3.21	−0.040
14.00–16.00	0.610	4.42	−0.080
16.00–18.00	0.771	2.48	−0.080
18.00–20.00	0.809	3.72	−0.040

naked eye scanning unless one overlapped the traces and made a systematic point-by-point analysis. The numbers from such an analysis, taken in 2 ms steps, is provided in Table 1. The first column lists the selected time segments. The second column shows the correlation between the 'without' and 'with' noise traces in each time segment, the third column the RMS amplitude differences in dB in each time segment, and the fourth column shows any shifts in ms between the two traces. The resolution here is 40 usecs per point.

One other difference must be addressed. Gobsch and Kevanishvili first collected emissions data without masking. Then they conducted their masking experiments and, at the end of the masking experiments, collected emissions again to clicks alone. They did not report any changes between the control conditions before and after the experiment. We have found that unless we alternated the conditions of testing (three conditions each of 'without' alternated with three conditions of 'with' masking) we would see a small but measurable change in the baseline of emission amplitude without any contralateral stimulation, which could have obscured our visualization of experimentally induced suppression.

8. Conclusions

We studied the suppressive effects of binaural, contralateral and ipsilateral white noise on linear TEOAE's. Binaural stimulation elicits the most suppression of otoacoustic emissions in a forward masking paradigm when the onset of the click train is 20 ms or less after the offset of a 408 ms white noise burst. Less suppression occurred to ipsilateral or contralateral stimulation, and the suppression essentially disappeared when the end of the noise was 100 ms or more away from the beginning of the click train.

Acknowledgements

NIDCD Center Grant P0 1 DC-000379, Training Grants T32-DC-00007, Department of Defense Neuroscience Cen-

ter Grant via N. Bazan, Kam's Fund for Hearing Research, The Kleberg Foundation, Lions' Eye Foundation and District 8-S Charities.

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